THE HAWAIIAN PLANTERS' RECORD

NEW FRUIT FLY IN HAWAII

This fly was discovered attacking mangoes in Honolulu, May 10, 1946. Surveys by several entomologists have since found it abundantly distributed all over Honolulu; in many widely separated parts of Oahu; in the Lahaina and Wailuku sections of Maui; and in many parts of the island of Hawaii. It attacks many kinds of fruits in a manner similar to that of the Mediterranean fruit fly and has a notorious record as a fruit pest in India, the Malay Archipelago, Formosa, and Saipan.

SECOND QUARTER 1946

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THE HAWAIIAN
PLANTERS' RECORD

A New Fruit Fly in Hawaii

By C. E. Pemberton

Several announcements of new insect pests appearing in Hawaii during the past two years have been made. Still another pest has been suddenly discovered and found well entrenched over much of the Territory. This is a fruit fly long recognized as a serious pest in many tropical regions. A discussion of its known distribution, host fruits, possible origin and discovery in Hawaii is given.

NEW FRUIT FLY IN HAWAII
Fig. 1.　Female of mango fruit fly *Dacus dorsalis* Hendel.

Early in May 1946, Mabel Chong of the Entomology department, Board of Agriculture and Forestry, reared some fruit flies from mangoes collected by her on Dole Street in Honolulu. These were quite different from any species previously known in the Islands, although they superficially resembled the common melon fly *Dacus cucurbitae* Coq. Entomologists F. X. Williams and D. T. Fullaway identified this fly as *Dacus ferrugineous* var. *dorsalis* Hendel. The writer sent specimens of this fly to C. F. W. Muesebeck, in charge of the Division of Insect Identification, U. S. Bureau of Entomology and Plant Quarantine, who reported in a letter dated June 20, 1946 that Dr. Alan Stone, Diptera specialist of the U. S. National Museum, examined the material and confirmed the identification by Williams and Fullaway; but that for certain clear-cut reasons the species should be known as *Dacus dorsalis* Hendel.

On May 25, C. G. Lennox, President of the Board of Agriculture and Forestry, called a meeting of the entomologists representing several institutions in Hawaii to plan and put into effect an immediate survey over the Territory to determine the present distribution of the fly in the Islands. Plant inspectors of the Board on Kauai, Maui, and Hawaii were also instructed to make similar surveys. These investigations soon established proof that the fly was well entrenched on Hawaii, Maui, and Oahu. On Hawaii, Wm. C. Look, Board Plant Inspector, found it in many widely separated sections where cultivated or wild fruits occurred. The fly had undoubtedly been on the island of Hawaii for many months at least. When Mr. Look was notified of the presence of the fly in Honolulu, he examined his field notes and insect collections and realized that he had actually bred this fly from waiawi fruits (*Psidium* sp.) which he collected at Mountain View, Hawaii on November 18, 1945, from fruits of mountain apples *(Eugenia malaccensis* Linn.) collected in the Hilo region, and from ripe bananas taken at Olaa during March 1946. M. Matsuura, Board Plant Inspector on Maui, found the fly at Lahaina and in the sand lots between Wailuku and Kahului. On Oahu, entomologists R. H. Van Zwaluwenburg, D. T. Fullaway, F. G. Holdaway, O. C. McBride, Q. C. Chock, and J. S. Rosa quickly determined the presence of the fly in many parts of the island and it was found to be very common in Honolulu.

To date, in Hawaii, it has been found infesting mangoes, ripe bananas, mountain apples, rose apples *(Eugenia jambos* Linn.), pomelo, and waiawi. It will undoubtedly be recorded from many other kinds of fruits growing in Hawaii as the studies proceed. The local fruit fly laboratory of the U. S. Bureau of Entomology and Plant Quarantine, under O. C. McBride, is undertaking a special investigation of this general subject. It is hoped that importation of known parasites of this pest occurring in other countries will be made as soon as possible.

The fly is known to occur in India, Ceylon, Malay Peninsula, the Philippines, Formosa, Java, Amboina, Bonin Islands, and Saipan. The principal fruits listed as being attacked by it are mango, orange, guava, papaya, coffee berries, rose apple, mountain apple, pear, peach, apple, quince, persimmon, plum, fig, apricot, banana, avocado, grape, sour sop, passion fruit, blackberry, loquot, chili pepper, and sapodilla. In several tropical regions it is known as the mango fruit fly. In Formosa it is sometimes referred to in literature as the citrus fruit fly and is a serious pest of oranges there.

It is probable this new fruit pest came to Hawaii in infested fruit carried by the crew or other passengers in planes from Saipan during the war.

The accompanying illustration shows the typical position assumed by the female fly when resting on a fruit.

Resistance to Termite Attack by Wood Treated with Copper Naphthenate

By C. E. Pemberton

On December 24, 1940 a local oil company submitted to the writer two pieces of northwest pine 36x1 1/3x3 1/2 inches, which had been impregnated with copper napthenate. It was suggested that wood so treated had merit in resisting termite attack. A two-inch block was cut from the end of one of the timbers. The freshly cut surface indicated that the compound had penetrated entirely through the samples. The two pieces were immediately taken to Waipio, Oahu and buried horizontally 4 inches underground in two places where the subterranean termite *Coptotermes formosanus* Shiraki was abundant and damaging a building adjacent thereto.

These samples were left in the ground until the latter part of March 1946 when they were removed, sectioned and examined. With the exception of a few small holes extending in a fraction of an inch and some slight surface scratching, the wood survived the test in almost perfect condition. Termites were still numerous in the soil around the timbers and a large colony was using the surface of one for a runway. Damage to untreated wood at the test spots was extensive throughout the period of the experiment. Though the soil was moist to wet much of the time, the two pieces of wood exhibited no signs of rot or other decay and when sectioned were found to be perfectly sound. A persistent odor suggestive of gasoline was noted in this wood at the time it was buried. After 5 years and three months underground in moist soil this emanation continued and appeared to be slightly intensified.

The above test was severe in every respect. In view of the results, timbers similarly termite proofed, if reasonably priced, should find a wide commercial use in Hawaii where the ground nesting termite causes extensive damage and large monetary losses to wooden structures in most of the Territory.

The condition of the timbers at the conclusion of the test is shown in the accompanying photograph.

The Influence of Certain Mineral Substances on the Quality of Sugar Cane

By R. J. Borden

No significant effects from various ratios of calcium, magnesium, and sodium were found on sugar cane juice quality as measured by its Brix, pol, purity and yield per cent cane. From different ratios of nitrogen and potash, however, juice quality was superior when high potash was associated with high nitrogen. From the evidence of interaction between the Ca-Mg-Na ratios and the N-K ratios, there is an indication that when potash was low a high calcium-low sodium ratio has produced a higher juice purity.

In discussions of factors which might be responsible for differences in the quality of sugar cane from irrigated lands, one of the questions raised has been "To what extent do changes in the ratios of calcium, magnesium, and sodium, which occur in the irrigation waters when associated with different levels of nitrogen and potash which are supplied in the fertilizers, influence cane quality?" Many of our irrigation waters add the various salts of calcium, magnesium and sodium in greater or lesser amounts to our cane soils and these salts can accumulate (temporarily at least) within the root zone. The application of soluble fertilizer salts of nitrogen and potash may further aggravate this undesirable soil condition, for their sum total effect could result in a total salt concentration which interferes with the osmotic absorption of water and essential nutrients.

The Plan

The present study,* which was in the nature of a skirmish test, was planned to find some of the effects on sugar cane when seven different ratios of calcium, magnesium and sodium were used in connection with three different ratios of nitrogen and potash. The soil used was a residual yellow-brown silty loam which had been formed under quite heavy rainfall conditions (150 inches yearly). It was purposely used because it is deficient in the basic mineral constituents we wished to study. It is quite acid in reaction (pH 5.4), relatively high in organic matter content, contains a fair amount of available nitrogen, and has high phosphate-fixing properties. An excellent granular structure makes it porous and well drained.

Single-eye cuttings of 32-8560 cane were planted in Mitscherlich pots holding 4000 grams of this soil, after a generous supply of a phosphate solution had been mixed in. Four replicates of seven different Ca - Mg - Na ratios with each of three N - K ratios, or a total of 21 different combinations of N - K - Ca - Mg - Na were provided as follows:

RATIOS USED (m.e. Basis)

Treatment No.	N - K - Ca - Mg - Na	Treatment No.	N - K - Ca - Mg - Na	Treatment No.	N - K - Ca - Mg - Na
A1	1 - 1 - 1 - 1 - 1	B1	2 - 1 - 1 - 1 - 1	C1	2 - ¼ - 1 - 1 - 1
A2	1 - 1 - 1 - ½ - 2	B2	2 - 1 - 1 - ½ - 2	C2	2 - ¼ - 1 - ½ - 2
A3	1 - 1 - 1 - 2 - ½	B3	2 - 1 - 1 - 2 - ½	C3	2 - ¼ - 1 - 2 - ½
A4	1 - 1 - ½ - 1 - 2	B4	2 - 1 - ½ - 1 - 2	C4	2 - ¼ - ½ - 1 - 2
A5	1 - 1 - 2 - 1 - ½	B5	2 - 1 - 2 - 1 - ½	C5	2 - ¼ - 2 - 1 - ½
A6	1 - 1 - ½ - 2 - 1	B6	2 - 1 - ½ - 2 - 1	C6	2 - ¼ - ½ - 2 - 1
A7	1 - 1 - 2 - ½ - 1	B7	2 - 1 - 2 - ½ - 1	C7	2 - ¼ - 2 - ½ - 1

All of these combinations were mixed into the soil at planting and also applied in solution to the surface at the ages of 2, 4, 5, and 6 months. The nitrogen was supplied in each instance from 2.0 grams of ammonium nitrate, the potassium from 4.3 grams of potassium sulphate, the calcium from 5.5 grams of calcium chloride, the magnesium from 6.0 grams of magnesium sulphate and the sodium from 2.8 grams of sodium chloride. The cane was grown under identical cultural conditions with position of replicates well balanced. From the data secured when the canes were harvested at the age of 12 months we have prepared the summaries and discussion which follows.

SIGNIFICANCE OF RESULTS

The data have been studied by analysis of variance to establish the significance of differences. The analysis of variance summary shows that there are some highly significant effects which are dominated by the N - K ratios. The effects from the Ca - Mg - Na ratios upon the total dry weight, pounds of cane, juice purity, and pounds of sugar, although significant with respect to error are not significant with respect to their interaction with the N - K ratios, but there are significant independent effects from the Ca - Mg - Na ratios on the per cent nitrogen and potash in the crusher juice and on the per cent moisture in the millable cane stalks.

ANALYSIS OF VARIANCE

		Mean squares or variance					
Source	D.F.	Total dry wt.	Lbs. cane	% moisture	Purity	Y%C	Lbs. sugar
N - K ratios........	2	629,538**	19.57**	9.58**	27.48**	9.43**	.594**
Ca-Mg-Na ratios....	6	109,140**	2.82**	3.59*	3.67	1.89	.099**
Interaction	12	65,686**	2.29**	2.67	5.45**	1.83	.059**
Error	61	23,123	.72	1.49	1.97	.99	.022
Coef. of Var.		12.2%	14.5%	1.7%	1.5%	6.6%	17.0%

ANALYSIS OF VARIANCE (continued)

			Mean squares or variances		
Source	Brix	Pol	%N in juice	%P2O5 in juice	%K2O in juice
N - K ratios..................	5.35**	9.89**	.006662**	.000260**	.102100**
Ca-Mg-Na ratios	1.89	2.62	.000200**	.000004	.000383**
Interaction	1.51	2.33	.000110	.000016	.000108
Error	.96	1.29	.000058	.000009	.000074
Coef. of Var.	4.7%	5.9%	27.6%	21.4%	10.0%

* Significant.
** Highly significant.

THE N - K EFFECTS

A summary of the independent effects from the three N - K ratios is given in Table I.

TABLE I
EFFECTS FROM N - K RATIOS
(Averages from 28 pots)

Measurement	Treatment A low N : high K	Treatment B high N : high K	Treatment C high N : low K	Minimum difference required
Total dry weight (gms.)............	1235	1406	1107	81
Pounds cane	5.88	6.66	4.99	.46
Per cent moisture	70.1	70.9	71.3	.7
Purity	94.0	93.2	92.0	.8

Y%C 15.5	15.2	14.4	.5
Pounds sugar .... .91	1.01	.73	.08
Brix of juice 21.1	21.0	20.3	.5
Pol in juice 19.8	19.6	18.7	.6
% N in juice .... .012	.034	.042	.004
% P205 in juice................... .018	.013	.012	.002
% K20 in juice .... .149	.083	.027	.005
% N in leaf-punch:			
at 4 months 1.74	2.12	2.19	#
at 5 months 1.71	2.01	2.07	#
at 6 months.................... 1.52	1.64	1.72	#
at 7 months 1.31	1.51	1.57	#

Not determined; only 1 composite sample.

With a high potash level (Treatments A and B) we note the following differences in the effects from the two levels of nitrogen: (a) no differences in the Brix, pol or Y%C; and (b) for the higher level of nitrogen, we have a lower purity and a lower concentration of both P205 and K20 in the juice, but higher yields of cane and sugar, a higher percentage of nitrogen in the juice and in the leaf-punch samples, a higher percentage of moisture in the cane and a greater total dry weight.

With a high nitrogen level (Treatments B and C) we have these differences in effects from the two levels of potash: (a) no differences in the per cent P205 in juice or in the per cent moisture in cane; and (b) for the higher level of potash, we note a higher Brix and pol, a higher purity and Y%C, more cane and sugar and total dry weight, a higher concentration of potash in the juice, but a lower percentage of nitrogen in juice and probably also in the leaf-punch samples.

The Ca - Mg - Na Effects

A summary of the three independent effects (on per cent N, per cent K20 and per cent moisture) from the seven ratios of Ca - Mg - Na is given in Table II; the data from these 7 treatments have been arranged in a descending order or rank for each measurement to facilitate their interpretation.

TABLE II

EFFECTS FROM CA - MG - NA RATIOS
(averages from 12 pots)

No.	Ca - Mg - Na Ratio	%N	No.	Ca - Mg - Na Ratio	% K20	No.	Ca - Mg - Na Ratio	% Moisture
6	½ - 2 - 1	.036	7	2 - ½ - 1	.099	7	2 - ½ - 1	72.0
7	2 - ½ - 1	.030	5	2 - 1 - ½	.091	5	2 - 1 - ½	70.9
5	2 - 1 - ½	.030	4	½ - 1 - 2	.088	1	1 - 1 - 1	70.6
3	1 - 2 - ½	.029	1	1 - 1 - 1	.087	4	½ - 1 - 2	70.5
1	1 - 1 - 1	.029	2	1 - ½ - 2	.086	2	1 - ½ - 2	70.4
4	½ - 1 - 2	.024	3	1 - 2 - ½	.084	3	1 - 2 - ½	70.4
2	1 - ½ - 2	.024	6	½ - 2 - 1	.080	6	½ - 2 - 1	70.4
Min. diff. req.		.006			.007			1.0

The interpretation of these data is not easy. Treatments 7 and 5 with their high Ca appear near the top of the list for all three measurements. However, in both of these treatments, but in the High N - Low K series only, the application of the salt

and fertilizer solutions at 5 months had seriously injured the cane in four of the eight pots.* In three of the pots from Treatment 7, the two primary stalks eventually died and in two of these pots no subsequent stalks came through to replace them; the third pot however did produce two good secondaries. In one pot only from Treatment 5, both of the primaries died, and only one secondary stalk took their place. Hence these abnormalities may account for the rank of these two treatments in Table II, and we should not put too much confidence in their being the actual effects of the associated mineral ratios.

Treatment 6 with its ½ - 2 - 1 ratio had the highest concentration of nitrogen in juice and was the lowest in potash. In general, the treatments with the higher Ca + Mg to Na ratio had a higher per cent N in juice than those with a lower Ca + Mg to Na.

The 2 - 1 - ½ ratio had a higher per cent K20 than the 1 - 2 - ½. In fact the two ratios with the high Mg had the lowest per cent K20 in juice, whereas the two with the high Ca had the highest per cent K20.

The only significant difference in moisture was the higher percentage in Treatment 7 which we have already noted as having some abnormalities that may have resulted from causes unknown.

There were no significant effects from the different Ca - Mg - Na ratios on the Brix, pol, purity, Y%C, or per cent P205 in juice.

The Interactions

Measured effects from the Ca - Mg - Na ratios which were indicated as not being independent of the influence from their N - K ratios are made a matter of record in Tables III and IV.

TABLE III

TOTAL DRY WEIGHT AND MILLABLE CANE
(Averages from 4 replicates)
(Differences required: Dry Wt. 215 gms.; Pounds Cane 1.60 lbs.)

Treat ment No.	Ratio of Ca - Mg - Na	Treatment A low N - high K grams dry wt.	lbs. cane	Treatment B high N - high K grams dry wt.	lbs. cane	Treatment C high N - low K grams dry wt.	lbs. cane
1	1 - 1 - 1	1248	5.84	1530	7.33	1257	5.87
2	1 - ½ - 2	1256	5.92	1390	6.50	1121	5.10
3	1 - 2 - ½	1236	5.85	1408	6.83	1309	6.05
4	½ - 1 - 2	1211	5.70	1353	6.33	1134	5.15
5	2 - 1 - ½	1208	5.77	1341	6.40	1095(a)	4.92(a)
6	½ - 2 - 1	1322	6.20	1403	6.60	1220	5.67
7	2 - ½ - 1	1161	5.89	1415	6.66	611(b)	2.19(b)

(a) 2 dead stalks, dry. (b) 5 dead stalks, dry.

* Without further verification it would be unwise to attribute the death of cane to Treatment 7 or Treatment 5 *per se*, since the cane in one of the four replicates of Treatment 7, and in three of the four replicates of Treatment 5 was not injured and made normal growth. We are at a loss to explain the discrepancy but suspect differences in soil moisture contents at the time the salts were applied.

TABLE IV

JUICE PURITY AND SUGAR FROM MILLABLE CANE

(Averages from 4 replicates)

(Differences required: Purity 2.0; Sugar .21 lb.)

Treatment No.	Ratio of Ca - Mg - Na			Treatment A (low N - high K)		Treatment B (high N - high K)		Treatment C (high N - low K)	
				purity	lbs. sugar	purity	lbs. sugar	purity	lbs. sugar
1	1	- 1	- 1	94.2	.91	93.4	1.14	92.0(c)	.84
2	1	- ½	- 2	93.8	.93	93.8	1.00	93.2	.77
3	1	- 2	- ½	94.2	.91	93.0	1.04	94.2	.96
4	½	- 1	- 2	94.1	.89	92.3	.94	92.5	.76
5	2	- 1	- ½	94.3	.91	93.3	.97	90.4(d)	.67(f)
6	½	- 2	- 1	94.6	1.01	93.3	1.00	92.0	.81
7	2	- ½	- 1	92.9(c)	.84	93.6	1.01	87.6(e)	.28(g)

(c) 1 stalk with dead top.
(d) Includes 1 young secondary stalk.
(e) Includes 2 young secondary stalks.
(f) Only 6 of initial 8 primary stalks.
(g) Only 2 of initial 8 primary stalks.

It is extremely doubtful that there has been any real interaction between the Ca - Mg - Na ratios and the N - K ratios, because most of the differences shown in Tables III and IV are due to differences between replicates with dead canes and those with sound canes. Until it can be more definitely shown that these dead canes were the effect of the known treatment applied, we should reserve an opinion in comparisons involving such cases. There is one place however, where an interaction may be suggestive. For instance, in juice purity, although there are no significant Ca - Mg - Na effects with the two series in which High K was used (Treatments A and B), the 1 - 2 - ½ ratio did have a slightly higher purity than the ½ - 2 - 1 when used with the low K series (Treatment C). This may indicate that with low potash, a prevalence of calcium over sodium is desirable for high juice purity.

Summary

The effects of different ratios of calcium, magnesium, and sodium when associated with different ratios of nitrogen and potash in the nutrients supplied to sugar cane have been recorded. The results are difficult to interpret correctly since the replicates did not always react alike to the known treatment applied, and some canes actually died. In such cases the specific effects of the mineral combination involved are probably masked.

The principal effects have come from the N - K ratios. We did not establish many clean-cut influences from the different Ca - Mg - Na ratios nor interactions between these two ratio groups.

The highest yields of total dry weight, of millable cane, and of sugar were generally secured from the High N - High K series, whereas most of the lower yields came from the High N - Low K series. The best juice purities and yield per cent cane were found in the Low N - High K series. Thus high potash stands out as being especially important in connection with both cane quality and yields.

A complementary effect in the absorption of nitrogen and potash by sugar cane is seen in the facts that (a) when the same amount of nitrogen was applied, the per cent nitrogen in juice and in leaf-punch samples was higher when the potash

supply was low; and (b) when the same amount of potash was supplied, the per cent potash in the juice was definitely higher when the nitrogen supply was low.

The Ca - Mg - Na effects suggest an influence on the per cent nitrogen and potash in the juice. The ½ - 2 - 1 ratio produced cane with a high concentration of nitrogen and a low concentration of potash in juice. The ratios with high Mg seem to have depressed the uptake of potash.

The Identification and Isolation of Molasses Constituents

By John H. Payne*

CONTENTS

Introduction

This investigation was undertaken to study two fundamental and interrelated problems:

1. The composition of final cane molasses.

2. Methods for the separation of the compounds in molasses in purified form.

The program was proposed by J. T. Phillips, President of the Pacific Chemical and Fertilizer Company, in 1939. Following an initial survey by C. S. Miner, the experimental work was begun in the laboratories of the Company early in 1941 under the direction of J. H. Payne. The work continued until the middle of 1943, with the exception of some six months following the outbreak of the war in December 1941, when the project was dropped in favor of more urgent war work.

Early in 1943 it became apparent that the most promising solution to the utilization of molasses problem lay in methods of processing cane juice which produced no "blackstrap" molasses. Consequently the study of molasses was suspended and all attention was directed toward the ion exchange treatment of cane juice.

Since work on molasses may not be continued for some time, it has seemed advisable to present the data at this time, in spite of the fact that some of the data are incomplete, some only fragmentary, and some ideas have not been tried experimentally.

The staff responsible for most of the laboratory work consisted at various times of Lawrence A. Boggs, Robert F. Gill, Jr., and Yoshimasa Ishihara, chemists, and Tatsuji Yamamoto, technician. As no one of these was on the staff throughout the investigation, the names of the chemists are indicated in the particular sections on which they performed a substantial portion of the work. In some instances these

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men did essentially all of the work in a section. Such is notably the case in the section on nitrogen compounds by Mr. Gill.

The facilities of the Experiment Station of the Hawaiian Sugar Planters' Association were made available at all times, and the splendid cooperation of the Director, Dr. H. L. Lyon, and his staff is gratefully acknowledged.

MOLASSES SAMPLE

Because of the wide variation in the composition of molasses from field to field and day to day, the selection of material for study required thorough consideration. It was the opinion, however, that the variations were for the most part quantitative rather than qualitative in nature. Furthermore, these quantitative differences would in most cases be within relatively narrow limits, and would rarely involve variations in order of magnitude. For this reason it was decided to select one sample of molasses that would have about the average composition for all Hawaiian molasses. The data from a thorough study of this representative sample would be of more value than data from a composite sample of many molasses.

A sample of final molasses was selected from Ewa Plantation Company. Its description follows:

The cane was from fields 50 and 81 of H 109 cane, second and fifth ratoons, 22 to 23 months of age. The fields were harvested on July 25, 1940. The massecuite from which the molasses was obtained was 100.9 Brix, and 51.25 apparent purity. The saturation temperature was 118° F., and the viscosity of crystal pressure filter sample was 1371 poises at 118° F. The molasses was collected on August 1, 1940, directly from the trough under the centrifugals. Two 55-gallon steel drums, fitted with removable covers, were collected and immediately put in storage at 37° F.

Analysis of the molasses by standard methods at the Experiment Station of the Hawaiian Sugar Planters' Association was as follows:

Total Solids	83.85	Glucose	23.51
Brix	95.82	Ash	11.86
Brix-Ref.	89.85	Glucose/100 Brix	24.54
Sucrose	30.92	Ash/100 Brix	12.38
True Purity	36.88	Glucose/Ash Ratio	1.98
Gravity Purity	32.27		

All subsequent work was done on this sample. Yearly analyses showed little change in composition, as shown in the following table:

ANALYSIS OF MOLASSES IN STORAGE
(In per cent)

	August 1940	July 1941	August 1942	July 1943	October 1944
Total solids	83.8	82.5	82.9	83.1	83.1
Ash	11.86	11.70	11.73	11.64	11.57
Sucrose (optical)	30.9	30.6	...	...	...
Sucrose (chemical)	...	32.1	31.6	31.3	31.9
Reducing sugars (Munson-Walker)	22.0	22.8	21.5	22.1	21.3
Glucose	10.0	9.6	9.1	9.2	8.9
Fructose	12.0	13.3	12.4	12.9	12.5

Inorganic Analysis
with Lawrence A. Boggs

Introduction:

Preliminary to a study of the organic constituents, a general picture of the inorganic composition was obtained by customary methods of analysis. Most important of the inorganic compounds present, of course, is water, the quantitative determination of which is not as simple as is sometimes assumed.

Total Solids:

The determination of total solids in molasses and other sugar-containing products is complicated by the thermal instability of the sugars and other compounds, possibility of chemical reaction between various chemical constituents at elevated temperatures, and by difficulties in dispersing the material to provide adequate surface for drying. With mixtures of such complexity it is likewise difficult to distinguish between "free" water and water of constitution. Decomposition is an important factor at temperatures above 70° C. At lower temperatures the rate of water removal is slow.

In the method commonly employed on Hawaiian molasses (1) the sample is dispersed on silica sand and dried for five hours in vacuum at 100° C. Decomposition is indicated by the continuous loss in weight after the initial five-hour period. The value obtained in five hours approximates values obtained at lower temperatures by approaching constant weight.

Evans and Fetzer (3) studied methods for the determination of moisture in corn syrups and found that the filter paper method was more rapid and more accurate. In this method the sample is dispersed by absorption on a coil of filter paper. The large surface area exposed makes for rapid drying at 70° C. in vacuum. Comparable results were obtained by dispersing the sample on Filter-Cel (2).

A comparison was made of the sand, filter paper, and toluene distillation (5) methods on the molasses sample after one year of storage. The results are shown in Table I.

TABLE I

COMPARISON OF METHODS FOR THE DETERMINATION OF TOTAL SOLIDS

(Molasses after one-year storage)

Method	Conditions (°C.)	Time (hours)	Total solids (%)
Sand	100 vacuum	5	82.43
			82.54
Sand	100 vacuum	7	81.71
			81.81
Sand	100 vacuum	11	80.57
			80.62
Sand	100 vacuum	16	78.31
Toluene distillation	112 vacuum	5	80.47
Toluene distillation	112 vacuum	8	80.28
Toluene distillation	112 vacuum	10	80.52
Filter paper	100 vacuum	7½	80.41
			80.51
Filter paper	70 vacuum	16	82.56
Filter paper	70 vacuum	20	82.49

The filter-paper method at 70° C. gives values identical with the commonly used sand method at 100° C. for five hours. The values become essentially constant in sixteen hours. Since little decomposition takes place at 70° C., it is believed that the values obtained by the filter-paper method at this temperature are a reliable measure of total solids in molasses, and this method was adopted, therefore, throughout the investigation.

Ash:

The carbonated ash was determined by ignition at 400° C. according to the method of the Association of Official Agricultural Chemists (4). The average value found was 11.86 per cent.

The carbonated ash was analyzed for principal constituents by the methods of the Association of Official Agricultural Chemists. The average results of duplicated analyses are shown in Table II.

TABLE II

ANALYSIS OF MOLASSES ASH

Constituent	Per cent of ash	Per cent of molasses
K_2O	40.5	4.80
CaO	10.50	1.25
MgO	6.38	0.76
$Fe_2O_3 - Al_2O_3$	2.54	0.30
SiO_2	3.90	0.46
P_2O_5	1.30	0.15
SO_3	12.67	1.50
Cl	21.6	2.57
As_2O_3		0.0002

Spectrographic Analysis:

A spectrographic analysis was performed by Paul Gow at the Experiment Station of the Hawaiian Sugar Planters' Association. The relative amounts of the elements were found to be:

Large Quantity: K, Ca, Mg, Fe, Al, Si
Small Quantity: Mn, Na
Traces: Ti, P, Cr, Co, Cu, Rb, Sr, Mo, W, Ba
Not Detected: Li, Be, B, F, V, Ni, Zn, Ge, Zr, Ag, Cd, Sn, Sb, Cs, Pt, Au, Tl, Pb, Bi

Suspended Matter:

The suspended matter in the molasses was determined by diluting a sample with an equal volume of water and passing it through a super-centrifuge rotating at 20,000 r.p.m. The sludge was removed from the rotor, washed twice with water, filtered, dried in vacuum at 70° C. for sixteen hours and weighed. The sludge was found to be 2.40 per cent of the molasses. Ash by the Association of Official Agricultural Chemists' method was 57.4 per cent, and the nitrogen content of the sludge was 0.58 per cent.

Ash analysis of the sludge ash was as follows by the Association of Official Agricultural Chemists' methods:

Constituent	Per cent
K_2O	16.1
CaO	13.9
MgO	1.36
Fe_2O_3	6.85
SiO_2	27.3
P_2O_5	1.45
SO_3	23.6

Additional data on the inorganic constituents will be found in the later sections on Solvent Fractionation, Ion Exchange Treatment, and Potassium Recovery.

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NITROGEN COMPOUNDS
with Robert F. Gill, Jr.

Introduction:

Of the many investigations of cane juices and molasses in the last fifty years, the few papers dealing with nitrogen contain only fragmentary information on the individuals and types of nitrogen compounds present. The commonly accepted picture is largely drawn from reiterated supposition of what is likely to be found in such a plant product.

Maxwell (30) in 1895 reported isolation of asparagine from Louisiana cane, but did not characterize it well. Beeson (4) in 1896 noted the presence of nitrogenous bodies which he thought to be amines. Knowledge of the nitrogen in some other plants was more advanced, so that reports on cane juices during this period (5, 41) began to use terms such as "albuminoids," "proteids," etc. In 1897-8, Shorey (43, 44) reported glycine in both the juice and molasses of Hawaiian cane. Zerban (70) later remarked that the reported glycine was probably an amide*. Shorey's further investigations led to reports of lecithins (42) in raw and clarified juices, syrups and molasses; and guanine (45) in juice and molasses. Guanine was well characterized. Peck (39) in 1906 isolated a crystalline substance analyzing 10.28 per cent nitrogen from Hawaiian waste molasses. This compound resembled aspartic acid, except that it was optically inactive.

In 1907 Browne and Blouin (11) published their long report on Louisiana cane and its products. This oft-quoted paper gives values for albumins and peptones,

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* Experience in this laboratory following Shorey's procedure would indicate a mixture of nitrogen compounds.

nuclein, etc., but these are empirical designations and do not demonstrate the actual presence of these types of compounds.

Zerban in 1912 made the nearest approach to a systematic fractionation of nitrogen compounds. Working with Argentine and Puerto Rico cane juices, 1-asparagine, d-glutamine and tyrosine were isolated. Recently, Jackson and Macek (20) accounted for a trace of Louisiana and Cuban cane nitrogen in the form of nitrogen-containing vitamins.

No general schemes for the fractionation of nitrogen compounds in sugar have been proposed. However, Vickery and coworkers (26, 37, 59, 64) have established some principles for methods applicable to complex plant products through their work with alfalfa and yeast.

The plan of investigation of the nitrogen compounds in Hawaiian cane molasses reported here was, first—to establish the relative distribution of the nitrogen in *types* of compounds and, second—to separate the nitrogen compounds from the great bulk of sugars and other interfering substances and to fractionate them into relatively simple groups from which individual constituents might be isolated.

The investigation has been limited to molasses, but the techniques developed (with certain indicated improvements) should be applied to a study of raw cane juice. Molasses does not represent the nitrogen of the cane plant, because of both losses and chemical change in the sugar-house process. This is illustrated in Table I which shows data for a series of raw juices which were limed (hot) to various pH levels. It will be noted that there is an average loss of over a third of the nitrogen through precipitation and volatilization. For a preliminary study, during which methods are developed, molasses offers the advantage of stability, in that a large homogeneous sample can be stored for a long time without appreciable change.

The Distribution of Nitrogen in Molasses:

1. *Introduction:*

The difficulty in working with molasses lies in the great complexity of uncrystallizable solids. The one-third to one per cent of nitrogen is vastly outweighed by the eighty to ninety per cent of other solids. Fortunately the method of Neuberg and Kerb (35) gives a means of separating the greater part of the amino-nitrogen compounds from non-sugars and inorganic salts. A newer tool, the ion exchange resin, is useful for the isolation of the nitrogen present in ionic form.

2. *The Total and Nitrate Nitrogen:*

Table II gives a series of values for the total nitrogen and the nitrogen-minus-nitrate contents of molasses. The methods are those of the A.O.A.C. (31, 32).

It has been more difficult in general to establish a value for the nitrogen content of whole molasses than for most of the fractions. This is due, apparently, to the small amount of nitrate nitrogen in the material in the presence of a substantial proportion of water. The conventional Kjeldahl "Total Nitrogen" (31) determination fails to pick up all of the nitrate in molasses solutions, so that the values average only 0.62 per cent. When, however, the samples are dried on the steam bath before adding the salicylic-sulfuric acid mixture, and the low-temperature heating continued for a few hours before high-temperature digestion, the nitrogen values are consistent and average 0.63 per cent. Reproducible figures are also obtained when the determinations are made upon powdered, dried molasses. The value of 0.63 per

cent has been taken as the true nitrogen content of the undried molasses.

The Kjeldahl "Nitrogen-Minus-Nitrate" (32) determination also gives reproducible values with dried molasses, but variable results are obtained with molasses solutions, showing that nitrate is not entirely eliminated under these conditions.

The difference between the two determinations on dried molasses established the nitrate nitrogen at 0.01 per cent. Nitrate was shown to be present qualitatively in molasses by applying the phenol disulfonic acid method for water analysis (48) to a distillate of molasses and ferrous sulphate, strongly acidified with sulfuric acid.

3. *Protein and Insoluble Nitrogen:*

Methods for the determination of what is conventionally termed "protein" in sugar products have appeared (1). However, these methods do not establish the presence of protein qualitatively or quantitatively. Such precipitants as the heavy metal salts and the oxides, picric and related acids, the complex acids such as phosphotungstic and phosphomolybdic acid, all in wide use, are in no sense selective for protein or related high-molecular weight split-products. It is a matter of conditions that determines how completely these will precipitate certain relatively simple nitrogenous compounds. On the other hand, salts like sodium and ammonium sulfates are useless for isolation of protein from complex, unknown mixtures, since many substances interfere with the salting-out process.

Hiller and Van Slyke (17) and recently Becker and Woldan (3) made systematic comparisons of the determination of protein in complex materials with a series of reagents, the former working with blood with and without nitrogenous additions and the latter studying animal feeds and feces. The common types of precipitants were represented by mercuric chloride, tungstic acid, metaphosphoric acid, colloidal iron, trichloroacetic acid, picric acid, alcohol, Baernstein's basic copper sulfate, uranyl acetate, and tannin reagents. Of these, only trichloroacetic and metaphosphoric acids showed promising selectivity for proteins without precipitating a large part of the intermediate split-products. Trichloroacetic acid is the more nearly specific. The action of metaphosphoric acid has been recently studied in detail (9, 10). The indications are that a union of a salt-like nature is actually formed with split-products down to low-molecular weight polypeptides. However, no data are given for the relative solubilities of the compounds formed.

In addition to the above, another precipitant, sulfosalicylic acid, has received some attention lately. It has been recommended (8) for removal of protein from samples without precipitating the tripeptide glutathione. Thus, the reagent is considered selective for protein.

Since there is no general agreement in the literature regarding the optimum concentrations of the three reagents for precipitating small amounts of protein, a series of determinations at varying concentrations was made with molasses solutions. All precipitations were in the cold. Separation and washing were accomplished by centrifuge to avoid filtration difficulties and adsorption errors. Precipitates were washed at least five times. During the operations the precipitates were in contact with either the reagent or the wash solution for about 24 hours, so that hydrolytic effect of any of the acid reagents might be noted.

The tannic acid precipitations were made as by Ambler and Byall (1) except that Gooch crucibles replaced filter paper, and the total nitrogen determinations were made by the conventional Kjeldahl method. The reagent was Baker C. P. tannic

acid. Blank determinations were run, substituting pure sucrose syrup for molasses, and the precipitation mixtures were heated for about two hours on the steam bath and filtered by vacuum, with difficulty.

None of the methods discussed consider the insoluble nitrogen of molasses. Insoluble, washed sludge amounts to approximately 2.5 per cent in the molasses studied. The insoluble nitrogen in this sludge does not necessarily come from the cane plant, but is possibly a soil constituent. Study of the washed sludge failed to suggest the presence of protein, although most color reactions are masked by soil. This insoluble nitrogen should be subtracted from the values obtained with protein precipitants.

The results listed in Table III show that the conventional tannic acid method precipitates much more than protein, and is of no value for determinations on mixtures containing protein split-products of intermediate type. Metaphosphoric, trichloroacetic and sulfosalicylic acids are seen to be similar to each other in action, except that hydrolysis by sulfosalicylic acid in the higher concentrations is apparent. This might be overcome by reducing the time of contact. Slight hydrolysis is also indicated in the precipitation with trichloroacetic acid at 10 per cent concentration. The misleading values obtained by measuring *initial* precipitation, a common practice, are shown in the last column of Table III for trichloroacetic acid.

Corrected for sludge nitrogen the protein nitrogen of molasess amounts to 0.9 - 1.0 per cent of the total nitrogen.

Qualitatively, the presence of protein in molasses was established with some difficulty, due to the small amount and the persistent dark color of molasses fractions. In those fractions concentrating the most complex nitrogen compounds, the color is also concentrated. Direct salting-out by Na_2SO_4, dialysis, and foaming all failed.

TABLE I

THE EFFECT OF HOT LIME DEFECATION ON THE NITROGEN CONTENT
OF CANE JUICES

	pH*	Nitrogen g/100 ml.‡	Nitrogen removed (%)
Raw	5.4	0.024	
Limed	10.0		
Clarified	8.4	0.012	50.00
R	5.2	0.032	
L	6.5		
C	6.0	0.028	12.50
R	4.9	0.042	
L	8.3		
C	7.4	0.020	52.38
R	5.4	0.048	
L	10.1		
C	8.7	0.024	50.00
R	5.1	0.017	
L	9.5		
C	8.5	0.013	23.53
R	5.3	0.034	
L	8.1		
C	7.6	0.029	14.71

R	5.0	0.038	
L	9.1		
C	8.3	0.015	60.53
R	4.9	0.014	
L	7.2		
C	6.3	0.011	21.43
R	4.9	0.021	
L	8.8		
C	8.1	0.015	28.57
R	5.0	0.021	
L	6.5		
C	6.0	0.017	19.05
R	4.9	0.023	
L	7.8		
C	6.7	0.013	43.48
R	5.0	0.018	
L	7.2		
C	6.3	0.011	38.89
R	5.2	0.023	
L	8.1		
C	7.5	0.015	34.78
R	5.0	0.026	
L	10.6		
C	9.6	0.018	30.77
R	5.2	0.019	
L	7.8		
C	7.1	0.012	36.84

* Beckman Type E glass electrode.   ‡ All determinations in duplicate.

TABLE II

NITROGEN IN MOLASSES

Sample	Nitrogen		
	Without Nitrate		Total
Dried Molasses	0.706		0.719
	0.706 Av. 0.71		0.716 Av. 0.72
Molasses Solution	0.605		0.604
(0.500g./ml.)	0.607		0.608
	0.603		0.621
	0.611		0.604
	0.602		0.613
	0.600		0.623
	0.604		0.604
	0.609		0.630
	0.609		0.644
	0.609 Av. 0.61		0.595
	.....		0.614
	.....		0.629 Av. 0.62
Molasses Solution dried	.....		0.625
before analysis	.....		0.632
	.....		0.631 Av. 0.63

method, however, is still in common use. Vondrak (67) and others, noting this error, employed the principle of Neuberg and Kerb (35) to separate the amides, ammonia, and most of the amino acids from the sugars. Browne and Zerban (12) give a detailed procedure for this method which consists essentially of alternate addition of 1N. mercuric acetate and sodium carbonate solutions to the sample, allowing one ml. of each per mg. of nitrogen, separating, washing the precipitate with water, and hydrolyzing with sulfuric acid. Sodium thiosulfate and magnesium sulfate are added with the magnesium oxide before distillation (in vacuo), to prevent interference by the mercury. Free ammonia is determined by distillation of the Neuberg precipitate without acid hydrolysis.

Table V gives comparative data on a series of determinations by the several methods discussed. Ammonia-freed titanium sulfate added before hydrolysis in one case (as an anti-humin catalyst) was of no appreciable benefit. The free ammonia of molasses is equivalent to only 0.95 per cent of the total nitrogen and may include some ammonia liberated from simple amides during the alkaline distillation. However, the residues after distillation were under pH 8.5, so that hydrolysis should be slight if asparagine were the amide. Glutamine in simple form should not be expected to survive to any large extent the milling process resulting in final molasses, to judge from the work of Vickery *et al.* (58) with asparagine and glutamine.

TABLE V

THE AMMONIA AND AMIDE NITROGEN

Sample	Method	NH_3 nitrogen %	Part of total N %	N in washed humin %
Soln. dried molasses	MgO wet distillation at atmospheric pressure	0	0	..
''	Ca $(OH)_2$ dist. in vacuo at 45-50°C. 1 hr.	0	0	..
''	20 hr. reflux with 10% HCl Ca$(OH)_2$ dist. in vacuo as above	0.08	11.1	45.8
''	''	0.07	9.7	44.4
Neuberg ppt. of soln. dried molasses	20 Hr. hydrolysis with 35% H_2SO_4 MgO dist. in vacuo, 45-50° C. 1 hr.	0.110	15.3	...
''	Same, but 15 hr. hydrolysis	0.113	15.7 Av. 15.5	...
Neuberg ppt. of soln. undried molasses	Same, 20 hr. hydrolysis but in presence 1 g. $Ti_2(SO_4)_3$	0.097	15.4	...
''	Same, but dist. without acid hydrolysis	0.005		...
''	''	0.004	Av.	...
''	''	0.007		0.95
''	''	0.006		...
Basic lead acetate ppt. of undried molasses	20 hr. hydrolysis with 35% H_2SO_4. MgO dist. in vacuo, 45-50°C. 1½ hrs.	0.011		...
''	''	0.011		1.7*

* 11.0% of Amide Nitrogen. Ammonia is excluded.

That some of the amide nitrogen is in higher complexes is shown by the last two determinations. In these, a basic lead acetate precipitate (washed with the precipitant) was substituted for the Neuberg precipitate. This contains 11 per cent of the amide nitrogen* and represents some 25 per cent of the (most complex) nitrogen of molasses. There are other indications of the complexity. For example, Amberlite IR-1 cation exchange resin consistently removed about 71 per cent of the nitrogen from whole molasses solutions, but only 35 per cent was recoverable on regeneration of the resin. The 35 per cent, representing the simpler nitrogen compounds of molasses** carried 43.9 per cent of the amide nitrogen (including ammonia). On the other hand, when a large Neuberg precipitate fraction of molasses was decomposed with hydrogen sulfide, 10.6 per cent of the molasses nitrogen, representing the most complex part, was tightly adsorbed onto the mercuric sulfide and could not be washed out‡. However, the amide nitrogen was recovered essentially completely. Arranging such data, the amide nitrogen can be bracketed roughly as follows, with regard to complexity:

The 10.6% most complex contains 00% of the amide nitrogen
     25.0%   ”   ”   ”   11% ”   ”   ”
     36.0%   ”   ”   ”   56% ”   ”   ”

6. *Polypeptide Nitrogen:*

There are several indications of the predominance of complex nitrogen compounds in molasses, for example, the low proportion of alpha-amino nitrogen (Table IV) in view of the small amount of protein (Table III). At the same time, the nitrogen precipitated by tannic acid (Table III) indicated high molecular weight split-products. The behavior of cation exchange resins with respect to molasses has been mentioned, as has the absorption of nitrogen onto mercuric sulfide in decomposition of a Neuberg precipitate of whole molasses.

In order to obtain further evidence, other precipitating agents were studied. Neutral and basic lead acetates when added to molasses solutions to maximum precipitation bring down a substantial proportion of molasses nitrogen, part of which is loosely adsorbed and can be washed out of the precipitates by a solution of the precipitant. Aspartic acid, but not asparagine, would be partly precipitated by the latter.

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* The reagent does not precipitate the free ammonia, like the Neuberg process.

** 71% - 35% or 36% is the complex part. Amide nitrogen in complex form is then 100 - 43.9 or 56.1%.

‡ Simple amino acids are not tightly adsorbed by the mercuric sulfide under the same conditions.

TABLE VI

NITROGEN PRECIPITATED BY LEAD ACETATE

Sample	Precipitant*	Wash‡	Filter or centrifuge	Nitrogen precipitated initially (%)	% of total N‡‡ Final
Soln. dried molasses	Dry basic lead acetate	....	F	58.3 av. of 3	
"	"	....	F	58.3 av. of 4	
"	"	B.L.5x	F	....	16.7**
"	"	" "	C	....	27.8
"	"	" "	C	....	23.6
"	Dry neutral lead acetate	N.L.5x	C	18.1	8.3
Soln. undried molasses	"	....	C	22.2	....
"	"	W 1x	C	....	15.9
"	"	" 2x	C	....	15.9
"	"	N.L. 1x	C	....	12.7
"	"	" 2x	C	....	9.5
"	"	" 3x	C	....	9.5
"	"	" 3x	C	....	7.9
"	"	" 4x	C	....	9.5
"	Dry neutral lead plus dry Na_2CO_3	....	C	22.2	....
"	"	....	C	17.5	....

* Added to solution to maximum precipitation.

‡ 15-25ml. portions: B.L.—Basic lead acetate, C.P., soln. of density 1.25; N.L.—neutral lead acetate. 3 H_2O, C.P., sat. solution; W—water.

** Actually composed of 13.9% adsorbed onto the PbS (when precipitate decomposed with H_2S and washed with H_2S water) plus 2.8% recovered in the deleaded fraction. Loss is apparent.

‡‡ Kjeldahl method for "Total N. minus nitrate" used here because of traces of nitrate in reagents. Calculations, however, are based on the total N of 0.63% for the undried molasses, which includes nitrate. The figures in the next to last column, therefore, should be slightly higher.

Data on a series of lead acetate precipitations are given in Table VI. *Initial* precipitation is measured by the nitrogen remaining in the centrifugate or filtrate after treatment with reagent, while the final is derived from nitrogen in the precipitate after washing with the indicated solution.

A reagent commonly used to characterize the bases in protein hydrolysates is phosphotungstic acid. When applied to whole molasses, over a third of the nitrogen is precipitated, which is many times the proportion of basic substance (free and combined) eventually found in molasses.

The procedure for the phosphotungstic acid separations was based on the average conditions used in the Van Slyke nitrogen distribution of protein hydrolysates (33),

in which the precipitation mixtures are 1N. in hydrochloric acid and contain 7.5 g. of phosphotungstic acid per 100 ml. of solution. The washing reagent was 0.4 N. in hydrochloric acid and contained 2.5 g. of phosphotungstic acid per 100 ml.

The precipitation mixtures were digested (closed) one hour on the steam bath, chilled ninety hours at 0° C. and separated and washed by centrifuge at 0° C. Each precipitate (25 g. of molasses) was washed four times with 15 ml. portions of washing reagent, followed by determinations of the nitrogen, with appropriate blanks on the reagents.

The results are given in Table VII. It is noteworthy that a basic lead acetate precipitate carried 13.9 per cent of the nitrogen in forms that could not be washed out of the lead sulfide when this precipitate was decomposed with hydrogen sulfide. This should be classed with the nitrogen adsorbed onto mercuric sulfide.

TABLE VII

PHOSPHOTUNGSTIC ACID PRECIPITABLE NITROGEN

Sample	Nitrogen (%)	Part of total N (%)	
1	0.223	35.4	
2	0.225	35.7	Av. 35.3
3	0.220	34.9	

The data of Table VII, which are not corrected for sludge nitrogen and any free bases, indicate that about a third of the molasses nitrogen occurs in the form of polypeptide and peptide complexes. An experimental method of measurement of this nitrogen was arrived at as follows:

If molasses were a mixture no more complicated than partially hydrolyzed protein or an animal tissue extract, the approximate proportion of the nitrogen in the form of polypeptides and related complexes might be demonstrated by measuring the amino nitrogen before and after hydrolysis. But the large proportion of sugars rules out this direct approach.

The plan of attack used in this investigation was to separate the nitrogen from the other solids by the Neuberg-Kerb principle, and to determine amino nitrogen on this relatively carbohydrate and salt-free fraction before and after hydrolysis. The procedure is attended by many difficulties and there are unavoidable losses which have to be taken into consideration.

A volume of whole molasses solution (0.500 g./ml.) was freed of insoluble nitrogen by centrifuge. The sludge was not washed, since it was found (Table III) there is but slight adsorption of nitrogen on these precipitates. A volume representing 25.00 g. was then freed of soluble protein by trichloroacetic acid in final concentration of 5 per cent. The precipitate was washed with 5 per cent reagent, and the centrifugate and washings were neutralized as soon as possible to prevent or minimize peptide hydrolysis. The solution was then reduced to 85 ml. in vacuo.

The concentrate was treated with 1N. mercuric acetate and 2N. sodium carbonate, allowing 1 milliequivalent of each per mg. of nitrogen, but in this case sufficient 95 per cent ethanol was added to the mixture to bring the alcohol concentration to 80 per cent. The addition of ethanol, according to Neuberg and Kerb (35), should insure essentially complete precipitation of the amino acids, excepting proline (imino group) and valine, the latter two of which may escape precipitation to the

extent of 25-30 per cent. The recently discovered diamino acid citrulline also should be included (68) with the losses.

The Neuberg precipitate was washed by centrifuge, as quickly as possible, five times with 80-85 per cent ethanol in 50-100 ml. portions, with protection from the air, because the glycine carbamide complex is subject to decomposition on standing, particularly in the presence of sodium carbonate. The washed precipitate was suspended in water, weakly acidified with acetic acid, warmed on the steam bath and decomposed with hydrogen sulfide, using motor stirring. The solution was decanted and the mercuric sulfide washed 5 times with very dilute acetic acid, the hot mixture being thoroughly resaturated with hydrogen sulfide each time. The separation and washing is tedious due to peptization of the sulfide. The sulfide was saved for nitrogen analysis.

After neutralization, the centrifugate and washings were concentrated in vacuo* and made up to 100.0 ml. A small amount of agglomerated mercuric sulfide was removed, washed five times and joined with the bulk of the sulfide above. A sample of the mercury-free fraction was analyzed for nitrogen to determine recovery. The amino nitrogen was determined on other portions. The nitrogen value at this point represents the original amino nitrogen plus any additional from partial hydrolysis due to conditions during preparation of the sample.

A 25.0-ml. portion of the fraction was then hydrolyzed by refluxing gently with 100 ml. of concentrated hydrochloric acid for 36 hours. The small sediment of humin was removed, washed five times, and was saved for nitrogen analysis. The centrifugate and washings were then concentrated in vacuo to remove most of the hydrochloric acid, and the remainder of the acidity was neutralized with sodium hydroxide**, after which the volume was brought to 100.0 ml. Small samples were taken for total and amino nitrogen determinations.

The procedure described was followed with one of the three molasses samples (No. 3), the fractionation of which is shown schematically in Plate I‡.

The polypeptide or peptide-complex nitrogen was calculated from the values given in Plate I, as shown in Table VIII, arriving at a figure of 31.6 per cent of the total nitrogen.

The partial hydrolysis of the sample during preparation is considered, as is the nitrogen adsorbed onto mercury sulfide. The amino nitrogen in the Neuberg filtrate fraction is negligible, but is listed since under some circumstances considerable amino nitrogen might escape precipitation. The humin nitrogen from hydrolysis of the mercury-free fraction is substantial (7.9 per cent), but it cannot be added to the other data of Table VIII with any certainty, since its source is not definitely know. The source of humin, in hydrolysis of protein free of foreign carbohydrate, is believed to be certain amino acids, but in this case it is not possible to estimate what proportion is formed from free amino acids and what part originates in complexes of the amino acids split during the hydrolysis.

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* Ammonia lost at this point.

** Further ammonia lost at this point.

‡ Sample 2 was a large sample to obtain fractions for other determinations. These alcoholic Neuberg fractionations, along with a series of aqueous fractionations, were to serve as pilot data for Part III of this report.

PLATE I

ALCOHOLIC NEUBERG FRACTIONATION OF MOLASSES

MOLASSES SAMPLE I

12.7% TOTAL NITROGEN

NEUBERG FILTRATE FRACTION

87.5% TOTAL NITROGEN

NEUBERG PRECIPITATE FRACTION

MOLASSES SAMPLE II

11.1% TOTAL NITROGEN

$\frac{0.1\%}{AMINO} N$

NEUBERG FILTRATE Hg-FREE

NEUBERG PRECIPITATE FRACTION

Hg-FREE 73.0% TOTAL NITROGEN

1.6% TOTAL NITROGEN

INSOLUBLE NITROGEN *

HgS

60.3% TOTAL NITROGEN

9.5% TOTAL NITROGEN

HUMIN

HYDROLYSATE[x]

(Diagram, rotated, showing nitrogen fractionation flow chart)

MOLASSES
SAMPLE III
0.9% OF TOTAL
NITROGEN
PROTEIN

1.6%
TOTAL NITROGEN
INSOLUBLE NITROGEN[*]

NEUBERG PRECIPITATE
FRACTION

10.6%
TOTAL NITROGEN
HgS

Hg-FREE
68.1% TOTAL
NITROGEN
15.9% AMINO[Δz]

7.9%
TOTAL NITROGEN
HUMIN

NEUBERG FILTRATE

41.7%
TOTAL
NITROGEN[x]
21.6%[z]
AMINO

HYDROLYSATE

* 1.6% of molasses Nitrogen lost when sludge not washed (Table III).

† All NH₃ lost, due to vacuum concentration over pH 7.

x Concentrate below pH 7 to avoid NH₃ loss.

Δ NH₃ also increased, due to partial hydrolysis during preparation of fraction, equivalent to liberation of 49.7% of amide nitrogen after correcting for free NH₃ of molasses.)

z % of Total Nitrogen

The principle of peptide estimation by increase in amino nitrogen does not take into consideration the part of the complex nitrogen in amide form, except for the liberated alpha-amino groups. Therefore a value for the ammonia liberated from complexes must be added, and this is obtained from cation exchange resin experiments*, as the amide nitrogen which was removed from molasses solutions, but not recoverable on regeneration of the resin. The value is conservative, since there is undoubtedly some hydrolysis of complexes during cation exchange treatment and regeneration.

The estimate 31.6 per cent is probably low, since it assumes all the amino acids liberated from peptide-complexes to be monamino, whereas diamino acids, nitrogen bases, and the imino substance proline may occur in such complexes in significant proportion.

TABLE VIII

MOLASSES POLYPEPTIDE NITROGEN

	% of total nitrogen	
Mercury-freed Alcoholic Neuberg Ppt. Fraction, Amino N before hydrolysis	13.9	
Less Amino N of whole molasses.................................. (Table IV)	9.2	
Peptide N split in preparing fraction................................	4.7	(Uncorrected)
Less Amino N in Neuberg Filtrate................................. (Escaped Neuberg precipitation)	0.1	
Peptide N split in preparing Fraction...............................	4.6	(Corrected)
Increase in Amino N on hydrolysis of alcoholic Neuberg ppt. Fraction, mercury free	7.7	
Nitrogen adsorbed onto mercuric sulfide............................	10.6	
Amide Nitrogen not recovered from cation resins on regeneration........	8.7	
Molasses Peptide-Complex Nitrogen................................	31.6	
Phosphotungstic acid Precipitate (Table VII), less free ammonia (Table V), Protein, Insoluble nitrogen (Table III) and actual basic N subsequently isolated *..	29.7	
Nitrogen removed from molasses by cation exchange, and not recovered on regeneration, less Protein and Insoluble nitrogen..............	33	

* Assuming all basic nitrogen to occur in free form, which may not be the case.

The magnitude, however, is confirmed by other approximate values (Table VIII), one derived from the phosphotungstic precipitable nitrogen and the other from cation exchange treatment of molasses.

7. *Betaine or Methylated Base Group:*

The proportion of nitrogen in the alcoholic Neuberg filtrate fractions (Plate I, samples 1 and 2) amounting to 11.13 per cent suggested the presence of methylated bases, since these are not precipitated by Neuberg reagent.

A phosphotungstic acid precipitation was made by the method previously described, employing an aliquot of a mercury-freed filtrate fraction (Plate I, sample 2), representing 37.50 g. of molasses. Because of the small bulk of the precipitate, a neutral solid diluent powder (Hyflo Super-Cel) was used to avoid possibility of any loss during manipulation. Only 0.3 per cent of the total nitrogen was

---

* Discussed later in this report.

found in the precipitate. A known sample of proline was not precipitated under the same conditions. As a check, samples of solutions containing approximately equivalent parts of betaine and choline were precipitated under identical conditions, and the bases completely recovered.

Therefore, the proportion of molasses nitrogen in the form of methylated bases is negligible. The small amount of basic nitrogen may represent bases other than the methylated type not precipitated by Neuberg's reagent, such as the dihydroxy pyrimidines.

8. *Non-Amino or Proline Nitrogen:*

The small proportion of nitrogen in the above fraction that is accounted for as nitrate nitrogen (1.6 per cent total nitrogen) and methylated base nitrogen (0.3 per cent) might point to amino acids not efficiently precipitated. However, a check on the amino nitrogen by the method previously described showed only a trace (0.1 per cent total nitrogen). This leaves 9-11 per cent of the total nitrogen as non-amino. This is likely proline but the actual identity is yet to be proved by isolation.

9. *Basic Amino Acid-Purine-Pyrimidine Group:*

The basic group was determined on a sample of hydrolysate of an alcoholic Neuberg precipitate fraction (sample 2, Plate I). The ammonia, which is also precipitated by phosphotungstic acid, was removed beforehand by distillation at 45-50° C. in vacuo in the presence of an excess of milk of lime, and the insoluble sediment was removed* and carefully washed. The phosphotungstic acid separation was made in the usual manner, employing Hyflo Super-Cel as a neutral diluent for the precipitate.

There was 3.2 per cent of the total molasses nitrogen found in the precipitate, representing the bases (other than methylated) both free and combined. There is, of course, the complex nitrogen adsorbed on the sulfide which may contain basic components. The nature of this complex nitrogen has not been investigated**.

The designation of this nitrogen as "Basic Amino Acid-Purine-Pyrimidine Group" is sound in that members of each type were eventually isolated or identified in molasses. In any case, the hexone bases are readily precipitated by the Neuberg process and the purines are of such low solubility themselves, that the simple mercury salts should be expected in the fraction, whether or not the two amino purines, adenine and guanine, form carbamido complexes. The pyrimidines are very soluble, but two of them, cytosine and 5-methyl cytosine have amino groups and should thus be expected to react with Neuberg reagent.

The Fractionation of Molasses Nitrogen and Some Constituents:

1. *Introduction:*

Because the process of working down a large sample of molasses is long and difficult, the starting of this work could not be delayed until the guiding information

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* This contained 2.5% of the total nitrogen, which, if not removed, would have been measured as basic nitrogen. Its nature is probably calcium salts of dicarboxylic acid and substances analagous to the so-called "acid soluble humin" of protein hydrolysates.

** To get at the bases in complex form, one might make a phosphotungstic precipitation of whole molasses (desludged), hydrolyze and then make a second precipitation after ammonia removal.

of Part I had been secured. It was not anticipated that about 1/3 of the nitrogen occurs in complex substances. Thus there were considerable losses of nitrogen that could not be washed out of various precipitates.

No general schemes, even those applied to the relatively simple hydrolysates of pure proteins, embrace all of the constituents. The problem usually calls for several distinct approaches, each aimed at high recovery of certain substances at the expense of others. But, because of the time involved in preparing sugar-free nitrogen fractions from molasses, this exhaustive repetition of operations is prohibitive. However, an attempt has been made to note and remark on those nitrogen compounds that should, in theory, be lost or altered by a given operation. In a few cases, where a nitrogen compound was strongly suspected of occurring in molasses, and a given operation should result in its loss, it was planned to make a special separation for this constituent.

2. *Three Major Nitrogen Fractions of Molasses:*

The plan for separating the nitrogen compounds from the sugars and partly resolving them called for the formation of three major fractions: Fraction I, precipitated by neutral lead acetate, was to contain the insoluble nitrogen, the protein and higher complexes, and much of the color. The sugars should be largely eliminated. Fraction II, precipitated by Neuberg reagent, was to carry the bulk of the remaining nitrogen, and should also be sugar free (and relatively salt free). Fraction III was to contain practically all of the original sugars and salts and only the nitrogen compounds not precipitated by Neuberg reagent or incompletely precipitated under the experimental conditions.

Pilot experiments were carried out with small molasses samples to furnish data for predicting recoveries at various points in the large-scale operations. The behavior of neutral lead acetate has been given (Table VI). Several alcoholic Neuberg separations have been given (Plate I). However, it was not practical to apply the *alcoholic* Neuberg precipitation on a large scale, and it would be also very difficult to wash the sugars out of the large precipitate with 80 per cent alcohol. Therefore, the precipitation was made in aqueous medium. The approximate recoveries to be expected under such conditions are indicated by the pilot experiments in Table IX, from which it appears three-quarters or less of the nitrogen is retained as compared with 87 per cent in 80 per cent alcohol medium.

TABLE IX

NEUBERG PRECIPITATION OF MOLASSES IN AQUEOUS MEDIUM

Sample	Wash	Nitrogen in precipitate (%)	Part of total*** N (%)	Nitrogen in filtrate (%)	Part of total N (%)
Solution	5x with	....	....	0.18	25.0
9.520 g.	75 ml.				
dried	portions				
molasses	H$_2$O				
"	"	....	....	0.17	23.6
Solution	1x with	0.45	71.4**	0.14*	21.4 Av.
7.500 g.	H$_2$O 3x			0.13*	
undried	ppt.				
molasses	volume				
Solution	....	....	....	0.16*	22.2
8.584 g. dried					
molasses					
"	....	....	....	0.18*	25.0

* Centrifugate samples not including precipitate washings. These indicate *initial* precipitation, rather than amount retained after washing precipitate.

** 71.4% represents retained N, approximate initial precipitation is 100-21.4 or 78.6%.

*** Based on Total N of 0.63%. Nitrate carried into Neuberg filtrate fraction makes values variable, as noted before.

The preparation of nitrogen Fractions I - III is given in detailed schematic form in Plate II. Nitrogen was determined in the final fractions. These figures and the pilot data were used to estimate approximate recoveries at various points in the scheme.

Fraction I concentrated much of the color, but the other two remained so dark that little advantage was gained. The most complex nitrogen compounds were probably lost in the lead sulfide precipitate, since considerable work with Fraction I itself failed to isolate protein by common methods. It was not considered worthwhile to investigate the fraction any further at this point, in view of the small proportion of the nitrogen represented.

Nitrogen Fraction III, more or less an unavoidable fraction, is a poor starting material for subfractionation, because of the large proportion of sugars and salts. Another way of getting at this nitrogen through ion exchange is discussed later. The possible constituents in Fraction III may be predicted from the original work of Neuberg and Kerb with solutions of individual nitrogen compounds. Aspartic and glutamic acids, cystine and tyrosine are precipitated practically completely in *aqueous* solution. Because of low solubilities the latter two are best recovered from the precipitate by decomposition with hydrogen sulfide in *acid* solution. Glycine, serine, phenylalanine, histidine, tryptophane, iso-serine, and the amino-sugar glucosamine, were completely precipitated only after addition of alcohol. Alanine, leucine, valine and proline were troublesome. The first two were thrown down nearly completely by alcoholic precipitation in most trials, but proline and valine (which are alcohol soluble), escaped precipitation to the extent of 25-30 per cent. This might be anticipated with proline, which contains no free amino group.*

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* We have not investigated the mechanism, but it is probable that the partial separation of proline results from formation of the normal mercury salt and depressing the solubility of it by alcohol.

12.75
MOLASS
IN 4-5 l. W

82.4 g. NIT
11% SF

18-22% OF TOTAL N

Wash 5x by supercentrifuge
with 1:10 dilution of
saturated $Pb(Ac)_2$.

8-10%
OF TOTAL N

H_2S into water suspension.
Neutralize with Na_2CO_3.
Supercentrifuge 4-5x.

Saturate with H_2S.
Neutralize with Na_2CO_3
Supercentrifuge 5x.

H_2S water wash
in supercent.
5x.

Saturate with H_2S.
Supercentrifuge
4-5x.

H_2S water wash
by supercentrifuge
5x.

PbS +ADS.MATTER
2.5-5% TOTAL N.

NORMAL SLUDGE
1.51% TOTAL N.

Vac. concentrate
to 2l.
Filter & centrifuge
off PbS.

Wash by
filter and
centrifuge

Reduce in vacuo and
make to 2l.

NITROGEN
FRACTION I
3.84% OF TOTAL N
16% NaAc
0.3% SUGARS
SOME SEDIMENT

NITROG
FRACTION
48.68% OF TO
3.2% OF ORIG.
0.0096 g. AS
19.15 l.

ER
GEN

NaOH to pH 7.0
Powd. neutral Pb(Ac)₂ to near max. pptn.
Supercentrifuge and add pb(Ac)₂
Supercentrifuge 4-5 times.

NaOH to pH 7.0
Filter and wash.

Pb
SALTS

90-92 %
OF
TOTAL N

11.36 kg. Hg(Ac)₂ in 60 l. H₂O.
11.90 l. 6N. Na₂CO₃.
Filter and
centrifuge.

H₂S resaturate and
supercentrifuge
until no further pptn.

22-25 %
OF
TOTAL N

Remove ppt.
by centrifuge.

NEUBERG PPT.
65-70% OF TOTAL N

Centrifuge
wash 3 times.
Save first washing.

23-25 %
OF
TOTAL N

H₂S.
Centrifuge

H₂S into water suspension.
Re-treat with H₂S,
separate and wash 5x by
centrifuge and filtration.

Vacuum
concentrate.

Wash

HgS & ADSORBED
MATTER
31-33% TOTAL N*

*By difference, and
including NH₃ losses.

NITROGEN
FRACTION III
10.16 % OF TOTAL N
19.20 l.

V
II
N
RS

PLATE III

RESOLUTION OF THE PROLINE FRACTION

Phosphotungstic filtrate from alcoholic Neuberg filtrate (plate I, sample 2), representing 37.50 g. molasses, 0.025 g N, excluding nitrate. Slight excess $Ba(OH)_2$, filter, wash.

Barium phosphotungstate

Slight excess H_2SO_4, filter, wash.

$BaSO_4$

Concentrate to syrup in vacuo. Add EtOH, filter. EtOH wash.

Sugars + salts

Remove EtOH in vacuo. Add excess $Cu(OH)_2$, shake. Filter, wash.

$Cu(OH)_2$

Vacuo concentrate to gum. Evaporate off twice each with dry MeOH and Me_2CO

H_2O

Dissolve in dry MeOH. Filter, dry MeOH wash.

Sugars, Cu_2O
Copper salts of amino acids.

Evaporate MeOH, add H_2O. H_2S, filter, wash.

Remove sulfur.

CuS

Acidify, HCl.
Slight excess *Bergmann reagent.†
Chill 2 hrs. at 0°C.
Filter (sintered glass), ice water wash.

S

Sugars

Acidified MeOH.

Concentrate in vacuo. (decomposition visible below 60°)

Add MeOH, filter, MeOH wash. (mostly soluble)

Remove MeOH in vacuo.
Add H_2O, NaOH until basic.
H_2S, filter, wash.

$Cr(OH)_3$

Acidify, vacuum concentrate
Remove sulfur.

S

Alternate solution and vacuum concentration in H_2O and MeOH.

Evaporate, dissolve and filter from dry EtOH 3 times.

NaCl, S

Gum

Dissolve in H_2O.
Slow evap'n at 70°C to constant weight.

0.1918 g.

* Based on equimolar ratio, assuming all N is proline N.
† 10g. ammonium rhodanylate in 60 ml MeOH, (unstable).

Inasmuch as the molasses precipitations were carried out in a concentrated solution of sugars and inorganic salts, the solubilities of the carbamido compounds might be expected to be depressed initially. However, in removing the sugars and salt by the three washings (Plate II), the overall effect may be very small. Nitrogen Fraction III, then, should contain any proline (hydroxyproline*), valine, and variable small proportions of the monoamino acids. The trace of methylated base nitrogen would also be present.

Nitrogen Fraction II should contain any free amides, dicarboxylic acids, basic amino acids, and most of the monoamino acids. The two aminopurines and two aminopyrimidines should also be precipitated as carbamido complexes. The two non-amino purines, xanthine and hypoxanthine would probably be precipitated as the normal mercury salts. The two non-amino pyrimidines would very likely escape precipitation. Any complexes** not held in Fraction I or lost on the sulfide would be present in Fraction II. Glycine would be destroyed (at least partly) by long standing of the alkaline Neuberg precipitate.

The scheme which is shown in Plate II could be modified to avoid the major losses and provide as sharp separation. Fraction I would include insoluble sludge nitrogen only. Fraction II would include protein, complexes and simple compounds, by *aqueous* Neuberg. Fraction II could be subdivided into: A, simpler substances recoverable on decomposition of the Neuberg precipitate; and, B, the more complex compounds in the sulfide, recoverable as simple substances by acid hydrolysis, followed by removal of mercury as the sulfide. Part B would be subjected to alcoholic Neuberg treatment to separate any liberated proline etc. from the other compounds.

3. *Nitrogen Fraction III:*

The large-scale fraction of Plate II was not taken for examination, partly because those constituents theoretically represented are not sharply separated from Fraction II by the aqueous Neuberg process, and partly because a large amount of inorganic salts had been introduced. In its place a sample of a mercury-freed alcoholic Neuberg filtrate fraction (sample 2, Plate I) was taken. The sample was in the form of a phosphotungstic filtrate, representing 37.50 g. of molasses and 0.025 g. of nitrogen, aside from nitrate.

The procedure, aimed at the isolation of proline, is given in Plate III, employing principles from the Towne (52) method of isolating hydroxyproline, proline and valine (as methanol-soluble copper salts), and Bergmann's (6) method of separating proline from other non-basic amino acids and hydroxyproline.

A very slightly colored, very hygroscopic, crystalline substance (0.1918 g.) was obtained. As proline, its weight would account for 0.023 g. of the 0.025 g. of nitrogen. The substance strongly resembled known proline in crystal shape, but was optically inactive. Identification as crystalline picrate or phosphotungstate failed, since known 1-proline did not form characteristic crystals in either of these derivatives under the same conditions.

A stable derivative for identification purposes was sought in proline-phenyl urea

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* Reported not occurring in plants (24).

** Complexes on hydrolysis may yield simple compounds not expected in a Neuberg precipitate. Some hydrolysis occurs during Neuberg separation.

(46). This derivative was found difficult to prepare in pure form on a semi-micro scale. The unknown, however, yielded no products that could be compared with those from known l-proline in melting point. The amount of isolated material was insufficient for further study.

There is some indication that the material isolated is a diketopiperazine or cyclic anhydride type of compound. During the alternate evaporations in methanol and water, and the three separations from dry ethanol (Plate III), very small amounts of yellowish gum were collected. The gum was readily soluble in methanol and ethanol, but not in water. Traces appeared on each alcohol evaporation, persisting even in the final sample crystallized from water. According to Dakin (13) this would indicate diketopiperazine formation. Osborne (38) and others have also noted the phenomenon in connection with the Dakin Butyl Alcohol method for fractionating amino acids. Proline forms cyclic anhydrides with itself or other amino acids when heated in butanol or other water-miscible solvents. The proline-like substance isolated, then, may be a lower cyclic anhydride or a mixture, with the gum representing secondary formation of higher anhydrides.

4. *Molasses Nitrogen Isolation by Ion Exchange:*

Another way of getting at this same part of molasses nitrogen represented by Nitrogen Fraction III, or an alcoholic Neuberg filtrate fraction, was found in cation exchange resin treatment of whole molasses.

A series of pilot runs was made to establish: (1) the optimum conditions, and (2) the nature and proportion of the nitrogen recoverable by this means. Following the preliminary runs, a large quantity of molasses was treated. Experimental work was as follows:

Amberlite IR-1, a synthetic resin, and Catex No. 12, a sulfonated coal cation exchange material were employed in 200 cc. volumes in 2.5 cm. diameter glass columns of conventional design. Hydrochloric acid was used to regenerate the beds and wash out the molasses nitrogen compounds taken up by the resins. The beds were always freshly regenerated before use, employing 200 ml. of 4 per cent acid, followed by 300 ml. of distilled water.

The flow rate for the run, regeneration and wash phases of the cycle was 20 ml./min.

In the first five runs (Table X) 15.00 g. of molasses in 750 ml. of water solution were put through the column, followed by 750 ml. of wash water. The amount of molasses treated was held at about half the rated capacity of the resin, on the basis of inorganic ash. Run No. 6 was identical, except that the weight and concentration of molasses were doubled.

The treated solutions (plus washings) from each run were concentrated and aliquot samples analyzed for nitrogen. The recoverable nitrogen was determined by analysis of aliquot samples of the acid regenerant solution (plus washings).

After one of the runs with each resin, the washing was continued beyond the normal regeneration-wash phase. The extra washings were collected in increments, concentrated, and analyzed for nitrogen.

Samples of new resins were analyzed for nitrogen on an approximate volume basis.

Amide and alpha-amino nitrogen were determined on samples of a recovered nitrogen fraction from one of the runs.

An aliquot part of the mercury-freed alcoholic Neuberg filtrate fraction of molasses (Plate I, sample 2) was also treated by Amberlite IR-1 and the recoverable nitrogen determined. Conversely, a sample of the recovered nitrogen fraction from one of the cation exchange runs was subjected to the alcoholic Neuberg process, and the filtrate fraction obtained was analyzed for nitrogen.

The results are partly presented in tabular form (Table X).

The concentration and weight of molasses used in runs 1-5 appear to leave a margin of safety, while the conditions of run 6 have reached the limit for Amberlite IR-1 and definitely exceeded it for Catex. Therefore the conditions of the large-scale isolation were established proportionately to runs 1-5.

The proportion of nitrogen recovered shows a tendency to increase slightly from run to run. The examination of the extended wash samples revealed very small amounts of nitrogen* proportional to the volume of wash water. The tendency toward increased recovery, then, is due to the leaching from the beds of accumu-

TABLE X
ION EXCHANGE ISOLATION OF MOLASSES NITROGEN
(in per cent)

Sample	Amberlite IR-1		Catex No. 12	
	Part of total nitrogen		Part of total nitrogen	
	removed	recovered	removed	recovered
1. 15 g. of molasses in 750 ml. of solution	(95.8)	31.8	74.6	20.7
2. "	71.4	34.9	71.4	23.8
3. "	69.9	34.9	....	22.2
4. "	69.9	36.5	63.5	22.2
5. "	71.4	38.1	....	....
Average	70.7*	35.2	69.8	22.2
6. 30 g. molasses in 750 ml. solution	65.0	33.4	50.8	17.4
Aliquot of mercury-freed Neuberg Filtrate Fraction (Plate I, Sample 2)	11.1**	....	....	
5000 g. molasses in 150 l. solution	29.5*****			
Alcoholic Neuberg Filtrate Fraction of Amberlite Recovered Nitrogen, Run 5, above	9.5			
Alcoholic Neuberg Filtrate Fraction of Catex Recovered Nitrogen, Run 4, above	9.5			
Amberlite Recovered Nitrogen, Run 6, above Amino N. in	11.1***			
" Amide N. in	6.8****			

* Omitting first value.

** Equivalent to 100% recovery of Nitrogen in sample.

*** Average of 4 values. Slightly higher than whole molasses, due to partial hydrolysis during preparation of fraction.

**** Represents 43.9% of molasses amide N. (Table V). Value probably increased by the partial hydrolysis.

***** Some Nitrogen lost on salts crystallized out. Represents pooling of the 50 runs. 30% of original ash remains.

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* It was found later that some of this nitrogen can be removed from Amberlite IR-1 by the action of concentrated acid.

lated nitrogen removed from molasses but not recoverable on ordinary regeneration.

Nitrogen analysis on the resins themselves showed Amberlite IR-1 and Catex to contain only 0.2 mg./cc. and 4.5 mg./cc. respectively. Thus, the contamination of treated solutions or recovery fractions with very fine Amberlite resin particles cannot introduce a significant error in nitrogen analyses.

Cation exchange treatments of a sample of an alcoholic Neuberg filtrate fraction of molasses resulted in complete recovery of the nitrogen. And, conversely, the alcoholic Neuberg filtrate fraction of samples of cation exchange recovered nitrogen contained 9.5 per cent of the original molasses nitrogen.

Thus, the nitrogen isolated from molasses by cation exchange includes essentially all of the nitrogen compounds of Nitrogen Fraction III, but freed of sugars.

Recovery of all the simple nitrogen compounds except nitrate ion of molasses by cation exchange is indicated by complete recovery of the free alpha-amino nitrogen and 43.9 per cent of the amide nitrogen. It was thought that the dicarboxylic amino acids, which are acidic, would not be taken up by the cation resins, but the recent report of Englis and Fiess (14) has shown that all amino acids are taken up by cation resins, and that removal is seriously decreased only when the acidity of the solution treated is increased to pH 3. The molasses had a pH of 5.8.

Following the preliminary runs, 5 kilos of molasses in fifty portions were treated by Amberlite IR-1 in a large column (1⅜″) containing 800 ml. of resin. The conditions of preliminary runs 1-5 were adjusted proportionally to the larger unit. The flow rate was advanced to 70-80 ml./min., proportional to the cross-sectional area of the bed.

The recovered nitrogen fractions were pooled, concentrated in vacuo to near dryness, dissolved in hot water and the humin and insoluble matter were filtered out and washed with boiling water. Two crops of salts were removed by concentration. These were carefully recrystallized and the mother liquors rejoined with the nitrogen fraction. The final fraction was adjusted to 1000 ml., and samples were taken for nitrogen determination.

The proportion of nitrogen recovered in the large-scale runs is somewhat lower than in the pilot trials. However, in removing about 1 kg. of salts, some nitrogen loss is unavoidable.

It was planned to prepare a large-scale alcoholic Neuberg filtrate fraction from the concentrated cation exchange nitrogen fraction, in order to isolate the proline-like substance in amount large enough for detailed study. This phase of the investigation has not been completed.

5. *The Sub-Fractionation of Nitrogen Fraction II:*

Eight liters of Nitrogen Fraction II were taken for sub-fractionation, leaving a reserve for future study. The scheme, a composite one, was adopted from principles used in the resolution of protein hydrolysates. The sample was given an initial sulfuric acid hydrolysis, first, to avoid complications arising from nitrogen complexes remaining in the material, and second, to eliminate chloride as far as possible as this interferes with butyl alcohol extraction.

The first step was a butyl alcohol extraction, based on the Osborne (38)

PLATE IV

SUBFRACTIONATION OF NITROGEN FRACTION II, PRIMARY

NITROGEN FRACTION II
6l. REPRESENTING
5326 kg MOLASSES
1634 g. N

Reduce in vacuo to 1 l.
Add 500g. H₂SO₄ and
reflux 36 hrs.

Remove SO₄⁻⁻ by hot saturated Ba(OH)₂.

(Accidental loss, maximum of 20%.)

Reduce in vacuo.
Ba(OH)₂ to faint acidity.

Reduce to 700 ml.

Make 3 extractions:
pass into 4 L of
n-butanol, add H₂O
as needed.

AQUEOUS
PHASE
(some
sludge)
Remove
butanol
in vacuo.
(Sludge
partly
dissolves)

Wash

SLUDGE

Lime above pH 7.
Reduce in vacuo.

→ NH₃

Let stand.
Suction filter.

25%
EtOH
wash.

Reduce to thin syrup.
Add ⅓ volume EtOH.

Wash.

LIME & HUMIN

Boiling H₂O wash
5-6 times.

Boiling H₂O
digest 5X.

Wash.

HUMIN

BaSO₄

(BUTANOL EXTRACT
proline monoamino
acids, base hydrochloride)

(Filter traces of aqueous phase.)

Distill extract
in vacuo.

(Recovered butanol)

Treat with H₂O.

(More H₂O insoluble
gum appears)
Dry in vacuo with
acetone.
Wash 6X with 25ml.
portions of 95% EtOH.

DIKETOPIPERAZINE
FRACTION

Contains
K, Ca, Ba
salts.

MONOAMINO
ACID FRACTION

(Most of color
soluble)

Reduce again.

Remove EtOH in vacuo.

HIGHER AMINO ACID FRACTION

25% EtOH wash.

...DRATED ACID FRACTION

Filter twice.

Dry in vacuo with acetone.

Treat with dry EtOH.

(several mg. very hygroscopic)

GUM

(More gum EtOH-Soluble H₂O-insoluble)

Add H₂O and remove EtOH in vacuo.

Lime to pH 7-10. Distill in vacuo.

NH₃

Treat: HCl to pH 2. Add 60 ml Phosphotungstic reagent, plus 10 ml excess. Digest on steam bath 1 hr. and keep at 0°C for 16-18 hrs. Chill to -3 to -5°C and suction filter.

Wash 4x with phosphotungstate wash. Chill below 0°C.

(Carries most of color.)

Grind 1X and 2X warm with saturated Ba(OH)₂. Separate by suction filter.

NaOH to pH 7. Ba(OH)₂ in slight excess.

H₂SO₄ in slight excess.

Dry in vacuo with acetone. Treat with dry EtOH.

(Little or no proline expected in Nitrogen Fraction II)

PROLINE FRACTION

wash.

H₂SO₄ to slight acidity.

wash.

BARIUM PHOSPHOTUNGSTATE

BaSO₄

Dry EtOH wash.

SALTS

Digest with sat'd Ba(OH)₂ 1 hr. on steam bath.

Sat'd Ba(OH)₂ wash.

Wash

BaSO₄

BARIUM PHOSPHOTUNGSTATE

FIRST BASE FRACTION

PLATE V

SUBFRACTIONATION OF NITROGEN FRACTION II CONTINUED

HIGHER AMINO ACID FRACTION
Includes any glycine, some bases, and most of any tyrosine.

10% Oxalic acid in slight excess.*

10% excess phosphotungstic reagent. Steam bath digestion 1 hr; cool at 0°C 44 hrs. Chill -5°C; suction filter thru hard paper.

Excess Ba(OH)₂ †

Excess H₂SO₄
Wash BaSO₄ ppt.

Reduce in vacuo to 300 ml.

Remove and wash fine solids.

Remove excess H₂SO₄ with Ba(OH)₂. Wash.

Wash

Ca + Ba OXALATES

Wash with phosphotungstate wash at -5°C.

Wash.

BARIUM SALTS

BaSO₄

DECOMPOSE PRECIPITATE AS IN PLATE IV

Reduce to small volume in vacuo.

SECOND BASE FRACTION
(About 3 times size of FIRST BASE FRACTION)

BaSO₄

BARIUM PHOSPHOTUNGSTATE

Wash 3 times 25% EtOH

Suction filter and wash 5x.

HgS

Adjust to 65 ml.

Add:
29 g. $K_3[Cr(C_2O_4)_3]\cdot 3H_2O$
55 ml. 2N. HCl.
Shake, let stand overnight.

Stir and add KOH to pH 8-9. Alternately add saturated K_2CO_3 and $Hg(Ac)_2$ until white ppt turns yellow. Centrifuge.

$BaSO_4$

Clouds on standing. Remove by centrifuge

120 ml EtOH slowly; shake. Let stand 2 days. Suction filter.

Wash with: 1:4 HCl:EtOH mixture.

Dry 1hr in vacuo, 70°C.

22.5 g.

GLYCINE SUBFRACTION °

Further haze. Shake and sample.

3,935 ml 0.72 g. NITROGEN

Add equal volume EtOH. Centrifuge.

0.045 g. NITROGEN

DISCARD

Determine nitrogen corrected for samples removed.

H_2S, etc.

Reduce in vacuo.

HgS

FINAL HIGHER AMINO ACID FRACTION

MgO to pH 7-8 (odor of NH_3) Filter; wash 5x with H_2O containing $Ba(OH)_2$.

$Mg(C_2O_4)_2$, $Cr(OH)_3$, etc.

$BaSO_4$

* H_2S at this point suggests cystine or related compounds, partly decomposed by earlier concentration with lime (plate II.)
† Large amount required due to HCl in phosphotungstic reagent. Use of H_2SO_4 in reagent would be advantageous.
x Neuberg separation was made to remove sodium which interferes with Bergmann-Fox separation.
△ Bergmann-Fox method. Conditions were adapted from protein hydrolysate analysis.
o Reagent and alcohol alone give crystalline precipitate.

modification of the Dakin method, which minimizes diketopiperazine formation. This gives two crude, conventional fractions:

A. Butanol solution of proline and monoamino acids (excepting glycine) and (in the presence of chloride) some bases as hydrochlorides.

B. Aqueous solution of higher amino acids, bases, and glycine.

From the crude fraction (A) were separated the nominal sub-fractions: Diketopiperazine, Monoamino acid, First Base, and Proline.

The Diketopiperazine sub-fraction separates when the butyl alcohol is removed and the residue put into water. The Monoamino acid fraction is separated (along with various salts) by its relative insolubility in alcohol. The bases are then separated as phosphotungstates, and Proline is the part of the residue which is soluble in alcohol.

From the crude fraction (B) were separated the Dicarboxylic acid fraction, Second Base, Glycine sub-fraction, and, Final Higher Amino acid fraction.

The Dicarboxylic acid fraction was separated from (B) as calcium salts insoluble in dilute alcohol, after Foreman (15). The Second Base fraction was removed by phosphotungstic acid, glycine was separated by the method of Bergmann and Fox (7), employing the glycine-selective reagent potassium trioxalotochromiate. The final Higher Amino acid fraction was the residue after removal of the other sub-fractions.

The detailed procedure for the subfractionation of Nitrogen Fraction II into the eight nominal sub-fractions is given in Plates IV and V.

The names assigned the final fractions are to distinguish them, and not to identify them as to their true components. Thus, the Proline fraction should contain little or none of this substance, unless it was liberated from complexes during the final stages of the preparation of Nitrogen Fraction II. The Glycine sub-fraction should contain glycine only to the extent it would not have been destroyed during the unavoidable long standing in the alkaline Neuberg precipitate during the separation of Nitrogen Fraction II (Plate II). Even though there was extensive, observable reduction of mercury in that precipitate, it is not strong evidence for the presence of glycine, since other easily oxidizable compounds may have been present.

The First and Second base fractions are not to be rigidly compared with similar fractions from protein hydrolysates, since bases other than the basic amino acids are to be expected from a plant or animal sample originating from other than an isolated protein.

The sub-fractions were examined, except for the Monoamino, Proline, and Final Higher Amino acid sub-fraction, as follows:

a. The Glycine Sub-fraction: The 22.5 g. of crystalline material was worked down for isolation of any glycine, as follows:

The Bergmann-Fox procedure (7) was followed in direct benzoylation of the complex with benzoyl chloride in the presence of magnesium oxide. No hippuric acid was formed. Therefore, the filtrate was made definitely alkaline with sodium hydroxide and the precipitated magnesium oxide and chromic hydroxide were removed and washed with boiling water. The filtrate and wash

were then concentrated to near dryness in vacuo, sodium hydroxide was added
to pH 8-9 and a further small amount of inorganic matter removed. The con-
centrated solution was then treated with benzoyl chloride in 3 to 1 molar
excess (based on 2 g. glycine), maintaining alkalinity with sodium hydroxide.
Again no hippuric acid was produced.

The results do not establish the absence of glycine in the molasses studied,
but, if present, it was destroyed in the preparation of Nitrogen Fraction II.
The precipitation of glycine as the trioxalatochromiate complex fails if the
solution contains calcium, barium or sodium ions. However, these ions should
have been essentially eliminated in the procedure (Plate V).

b. Resolution of the Dicarboxylic Acid Sub-Fraction: Of the naturally oc-
curring amino acids, the lime-alcohol precipitate may contain tyrosine and
cystine in addition to the dicarboxylic acids. Yet Foreman reports little dif-
ficulty in the examination of protein hydrolysates, unless the sample was high
in tyrosine. Extra steps were taken (Plate IV) in the separation of the di-
carboxylic acids to effect as sharp a fractionation as possible.

The detailed procedure for resolution, given in Plate IV, is essentially that
of Foreman.

The treatments of the lime-freed solution first with silver sulfate and then
with phosphotungstic acid were found necessary by Foreman even with hy-
drolysates of relatively pure proteins. However, he did not determine the
nature of the basic substance. In the case of molasses it would have been wise
to save the very small phosphotungstic acid precipitate for examination, in
view of the fact that purines were later isolated* from the base sub-fractions.
Tyrosine did not appear at any point during the sub-fractionation.

The 12.3 g. of clear, glass-like solid (Plate VI) is definitely not a measure of
the dicarboxylic acids, because of contamination with inorganic salts. This
solid eventually yielded three components: 3.19 g. of crude aspartic acid and
5.66 g. of crude glutamic acid, plus another approximate 2.5 g. (corrected for
0.26 g. ash) in the form of pyrrolidone carboxylic acid. All were clear, glass-
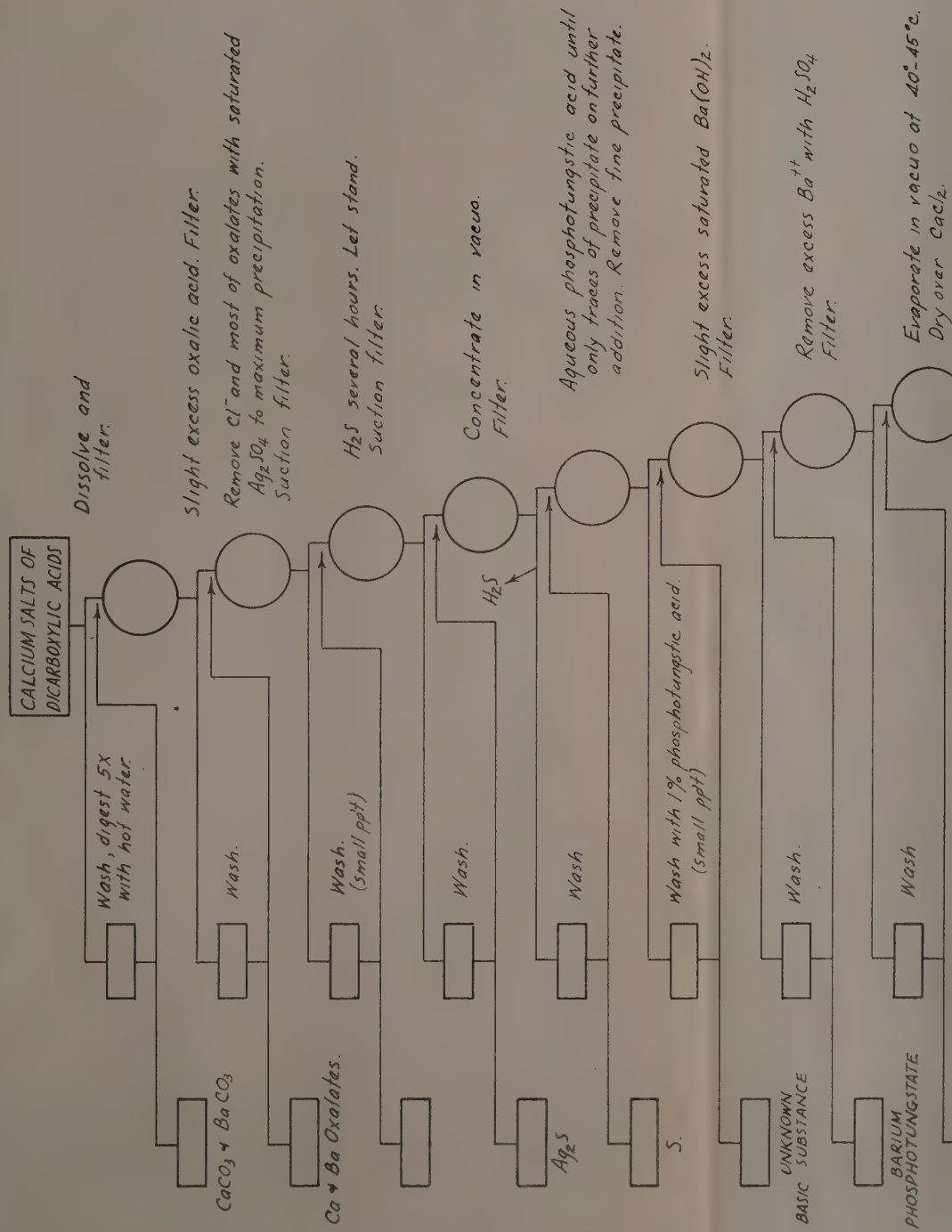like solids of very light amber tint, but exhibiting no tendency to crystallize.

c. The Crude Aspartic Acid: The crude product, to judge from the isolation
steps, should represent a fairly distinct substance, readily purifiable by re-
crystallization. However, only 1.68 g. of aspartic was ultimately obtained, and,
it was very difficult to crystallize the crude material, due to the persistent
presence of an unknown inhibiting substance. Despite the low solubility of
aspartic acid, the substance formed solutions of the consistency of honey.
When the inhibiting substance was finally eliminated from part of the crude
product, the behavior became normal. The isolation was carried out as follows:

The crude product was dissolved in the minimum amount of water. After
the usual methods failed to induce satisfactory crystallization in the viscous

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* Guanine is alkali-soluble, but lime-alcohol separation might have been under the critical
pH. The second base fraction might have been advantageously separated before the dicar-
boxylic acids.

PLATE VI

SUBFRACTIONATION OF NITROGEN FRACTION II, CONTINUED.
RESOLUTION OF DICARBOXYLIC ACID SUBFRACTION

CALCIUM SALTS OF DICARBOXYLIC ACIDS

Dissolve and filter.

Wash, digest 5x with hot water.

CaCO₃ + BaCO₃

Slight excess oxalic acid. Filter.

Wash.

Ca + Ba Oxalates.

Remove Cl⁻ and most of oxalates with saturated Ag₂SO₄ to maximum precipitation. Suction filter.

Wash. (Small ppt.)

H₂S several hours. Let stand. Suction filter.

H₂S

Wash.

Ag₂S

Concentrate in vacuo. Filter.

Wash

S.

Aqueous phosphotungstic acid until only traces of precipitate on further addition. Remove fine precipitate.

Wash with 1% phosphotungstic acid. (small ppt)

UNKNOWN BASIC SUBSTANCE

Slight excess saturated Ba(OH)₂. Filter.

Wash.

Remove excess Ba⁺⁺ with H₂SO₄. Filter.

BARIUM PHOSPHOTUNGSTATE

Wash

Evaporate in vacuo at 40°-45°c. Dry over CaCl₂.

BaSO₄

12.5 g. — Transparent, light amber solid. No crystals.

Grind 3-4 X with glacial acetic acid. Filter by suction. Wash with glacial acetic acid.

Remove acetic acid with ether.

Evaporate in vacuo and dry over CaCl₂.

Dissolve in 1½ l H₂O. Excess Cu(OH)₂. Stir. Suction filter, wash 5X with warm water

Reflux 36 hrs with 10ml HCl. Filter and wash trace of humin.

Treat with Norit A and wash.

2.48 g.

C

Evaporate in vacuo to 5ml syrup. Saturate at 0°C with dry HCl. Let stand, filter, wash with cold HCl. Repeat.

0.26 g. BaCl₂

Evaporate to heavy syrup. Saturate with HCl. Let stand at 0°C.

PYRROLIDONE CARBOXYLIC RESIDUE

(Light blue needles in mass)

Chill overnight at 0°C. Filter Wash

Reduce to 11 in vacuo at 40°-45°C. Chill, filter crystals and wash.

Remove CuS, with H₂S. wash.

Reduce to 150ml. Remove and wash CuS.

CuS.

Resaturate with H₂S. Treat with Norit A. Filter and wash.

Suspend in 100ml H₂O. Decompose with H₂S. Remove CuS. Wash

Evaporate in vacuo to dark amber syrup. Dissolve in H₂O and decolorize with Norit A. Filter and wash.

Reduce to 500ml. Remove sludge, crystals (3 mg.)

Retreat with Norit A. Filter and wash

Light amber syrup. Reduce in vacuo.

CuS.

Evaporate in vacuo. Dry.

C

3.96 g.

CRUDE ASPARTIC ACID

5.66 g.

CRUDE GLUTAMIC ACID

solution, an equal volume of ethanol was added and the mixture was shaken for several hours. A gummy solid appeared, which after a few days formed a small bulk of off-white nodules and poor crystals. This was filtered off, washed with 25 per cent and 95 per cent ethanol and dried over phosphorus pentoxide, yielding 0.318 g. The mother liquor gave a second crop of 0.0851 g. The alcoholic liquor was saved.

The two crops were recrystallized twice, decolorizing with a little Norit A. The mother liquors were discarded. The recrystallized material was composed largely of poorly shaped crystals in comparison with known l-aspartic acid. A semi-micro Kjeldahl determination gave a value of 8.18 per cent nitrogen which corresponds to 77.7 per cent purity. A micro ash determination showed 12.6 per cent which consisted of calcium and silica. Discounting the ash, the purity is raised to only 90 per cent.

The alcoholic mother liquor was freed of alcohol, decolorized again, and a reseparation as the copper salt was made as follows: Excess copper hydroxide was added to the boiling solution. The acidity seeming high, dilute sodium hydroxide was added dropwise until, at pH 4, needles of copper aspartate appeared in great bulk. Addition of alkali was stopped at pH 6, since the solution was apparently buffered on the acid side. Crystallization appeared to be complete since the copper ion color was no longer detectable in the solution. After chilling, the mass of blue needles was filtered off and washed thoroughly with cold water. Decomposition in the usual way with hydrogen sulfide gave a nearly colorless solution, after removal of traces of colloidal sulfur. On concentrating the solution in vacuo, crystals appeared while the volume was still large. The first crop weighed 0.8611 g. On reducing nearly to dryness a second 0.4111 g. crop, probably less pure, was obtained.

Microslide recrystallization of a sample from the larger crop yielded a greater proportion of perfect crystals than could be obtained with the Eastman White Label product (assay 97.5 per cent) under the same conditions. Assay of the larger crop showed the sample to be practically pure.

d. The Pyrrolidone Carboxylic Residue: This crude product was estimated to be equivalent to approximately 2.5 g. of glutamic acid. However, only two small crops, one of 0.314 g. and a second of 0.2 - 0.5 g. were obtained. The same difficulty encountered with the crude aspartic acid was experienced in the crystallization of glutamic acid as the hydrochloride. The melting point of the hydrochloride was low in comparison with values in the literature (33, 47). Because the benzoyl derivative is reported as having a definite melting point (46), an attempt was made to benzoylate the second crop and the mother liquor from which the two crops were taken. The preparation of the derivative was unsuccessful.

The pyrrolidone carboxylic acid hydrolysate (Plate VI) gave a small crop of crystals after standing at 0° C. for four days. This was filtered off through sintered glass, and washed with chilled, concentrated hydrochloric acid when some redissolving was apparent. After drying 2½ hours in vacuo at 70° C., the crop weighed 0.0314 g. and had an off-white color. The crystals were

comparable with those prepared from Eastman White Label d-glutamic acid. The melting point was observed as:

Softened	All melted*
187°	195°

The mother liquor and washings from the first crop were worked down again. Crystals appeared in a few days and growth was increased by a second saturation with dry hydrogen chloride. A third saturation after a month of standing at 0° C. resulted in a nearly solid mass of crystals through the very viscous liquid. After about two weeks of standing, the crystals were quickly filtered off through sintered glass, protecting them from atmospheric moisture. The crop was washed well with chilled concentrated hydrochloric acid. The yield was 0.2 to 0.5 g. and off-white in color. The mother liquor and washings were concentrated in vacuo several times with the addition of water to remove most of the acid. Much of the developed color was removed by treatment with Norit A in the cold. Attempts to prepare the benzoyl derivative from the second crop and the mother liquor failed. This was also the case with known d-glutamic acid, both by the well-known Schotten-Baumann procedure and the Fisher modification which substitutes sodium bicarbonate for the stronger alkali.

e. The Crude Glutamic Acid: In working down the crude product, difficulty in crystallization was encountered more pronounced than that encountered with the crude aspartic acid. In this case, no method of eliminating the inhibiting principle was found. Careful elimination of the ash was of no detectible benefit. Only a small proportion of the crude product (18.8 per cent) was eventually accounted for as glutamic acid hydrochloride. The procedure was as follows:

The crude glutamic acid was dissolved in the minimum volume of concentrated hydrochloric acid, saturated with dry hydrogen chloride and held at 0° C. for two weeks with daily shaking, without the appearance of definite crystals. A milligram or two of prepared d-glutamic acid hydrochloride was added as seed, which appeared to have a slight effect. After another two weeks the mixture was resaturated with hydrogen chloride, but after four months standing at 0° C. with occasional shaking only a small amount of solid phase had accumulated. This was separated by a pre-chilled, sintered glass filter, and washed with chilled concentrated acid. The bulk of the solid was dark and proved to be inorganic with a light sprinkling of off-white organic crystals.

The dark mother liquor and washings were neutralized with sodium hydroxide, and, with motor stirring, saturated mercuric acetate was added to definite excess (300 milliequivalents), keeping the mixture alkaline with 1 N. sodium carbonate. After standing overnight, the precipitate was separated and washed by centrifuge, using Hyflo Super-Cel as a neutral diluent, until the washings were chloride free.

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* Melts with bubbling, leaving an apparently dry residue when all gas evolved. Values uncorrected, capillary tube, copper block method.

The washed precipitate was decomposed in the usual way, and the last haze of sulfur was removed from the mercury solution by filtering through Hyflo Super-Cel. Twenty ml. of concentrated hydrochloric acid were added to prevent pyrrolidone carboxylic acid formation, and the 250 ml. of solution was concentrated in vacuo at 50° C. At a volume of 3-4 ml., more acid was added to maintain the acidity and the solution was again reduced to a light amber-colored syrup. This syrup was saturated with dry hydrogen chloride at 0° C. and allowed to stand. After five months crystallization was not very extensive. Another four months of standing gave some increase. The crystals were separated and washed in the usual way. After drying over phosphorus pentoxide, the crop weighed 0.0937 g. and was off-white in color. The mother liquor and washings were made up to 100.0 ml. and the total nitrogen was determined in triplicate.

The melting point of the crystalline material was taken by the same method used on the sample from the pyrrolidone carboxylic acid hydrolysate (sample 1, Table XI).

The crystal crop was dissolved in water, warmed, treated with a small amount of Norit A, and filtered through Hyflo Super-Cel. The filtrate and 6 N. hydrochloric acid and water washings were evaporated down by suspending over calcium chloride, followed by drying over phosphorus pentoxide in partial vacuum. The melting point was determined again (sample 2, Table XI).

A sample of known hydrochloride was prepared from Eastman White Label d-glutamic acid, dried in the same manner. The melting point is given as sample 3 in Table XI.

The unknown and known crystals (sample 2) were then ground together in approximately equal weights, and the melting point of the mixture was determined (sample 4, Table XI).

TABLE XI
MELTING POINT OF GLUTAMIC ACID

Sample no.	Starting temp.*	Shrink*	Rapid melt*
1	180° C.	188° C.	189 complete below 190° C.
"	185	190	191
2	185	188 plus	189.5-190
"	186	190	191
3 E. K.	182-3	192-3	194
" "	186	192	196
4 Mixed E. K.			
& 2	184	189 plus	191 plus

* Approximate stem correction to be added 4°.

The nitrogen analyses of the mother liquor and washings revealed nitrogen equivalent to 1.239 g. of glutamic acid hydrochloride. Adding the 0.0937 g. in crystalline form, 1.33 g., or 18.8 per cent of the crude glutamic acid as hydrochloride is accounted for. Under the microscope the isolated crystals, like those from the pyrrolidone carboxylic, were comparable with the known substance, but exhibited a higher proportion of irregular shapes. The identity is established by the melting-point data, but a persistent trace of impurity is evident.

The melting point of the crystals from the crude glutamic acid was sharper than obtained with the product from the pyrrolidone hydrolysate.

The crystal-inhibiting impurity was evidently not decreased along with the ash on reprecipitation by the aqueous Neuberg process. Because of this, there is a possibility that another amino acid is responsible for the poor yield. Betahydroxyglutamic acid, the less well-known member of the naturally occurring dicarboxylic acid group, is a possibility. However, insufficient sample was available for further study. A similar inhibition in crystallization of glutamic acid in soy bean oil residues has been reported (34), but the nature of the impurity was not ascertained. Some promise for future application is given by the recently reported isolation method of Opsahl and Arnow, (36) based on extraction of practically pure glutamic acid from solution by ethyl acetate.

f. Resolution of the First and Second Base Sub-fractions: No generally *inclusive* scheme has been proposed for the separation of a complicated mixture of nitrogen bases from plant or animal matter into relatively distinct groups. The only approach to such a scheme was outlined by Vickery (55) based principally upon work on the basic nitrogen of alfalfa and yeast (26, 59-64). Therefore, the known, non-alkaloid plant bases which might be encountered are discussed below in relation to Nitrogen Fraction II of molasses and to their probable behavior in a scheme for their resolution.

Bases Possibly Present: The methylated bases are excluded from Nitrogen Fraction II, since they are carried into the Neuberg filtrate. The three common basic amino acids or hexone bases, arginine, histidine and lysine, and the sulfur-containing cystine (or cysteine) may be expected. The rarer basic amino acids, hydroxylysine (54) and canavanine (23), but not citrulline (68), should be included. All of the four purines originating in nucleic acids should be in the fraction. Accompanying these as nucleic acid split-products, the two amino hydroxy pyrimidines should be expected, but the two dihydroxypyrimidines would not be precipitated by the Neuberg process*. Ethanolamine, another nucleic acid degradation product would not appear in Nitrogen Fraction II. Of the miscellaneous bases, glucosamine and guanidine would be included, the latter as a decomposition product of arginine or guanine. Guanidine, however, is easily hydrolyzed by alkali and heat. The nitrogen-containing vitamins are present in very small amounts, as reported later.

Potential Losses: The loss of a quarter of the molasses nitrogen by absorption onto lead sulfide and mercuric sulfide represents a selective loss of complexes. These might conceivably include basic components not occurring in simple form.
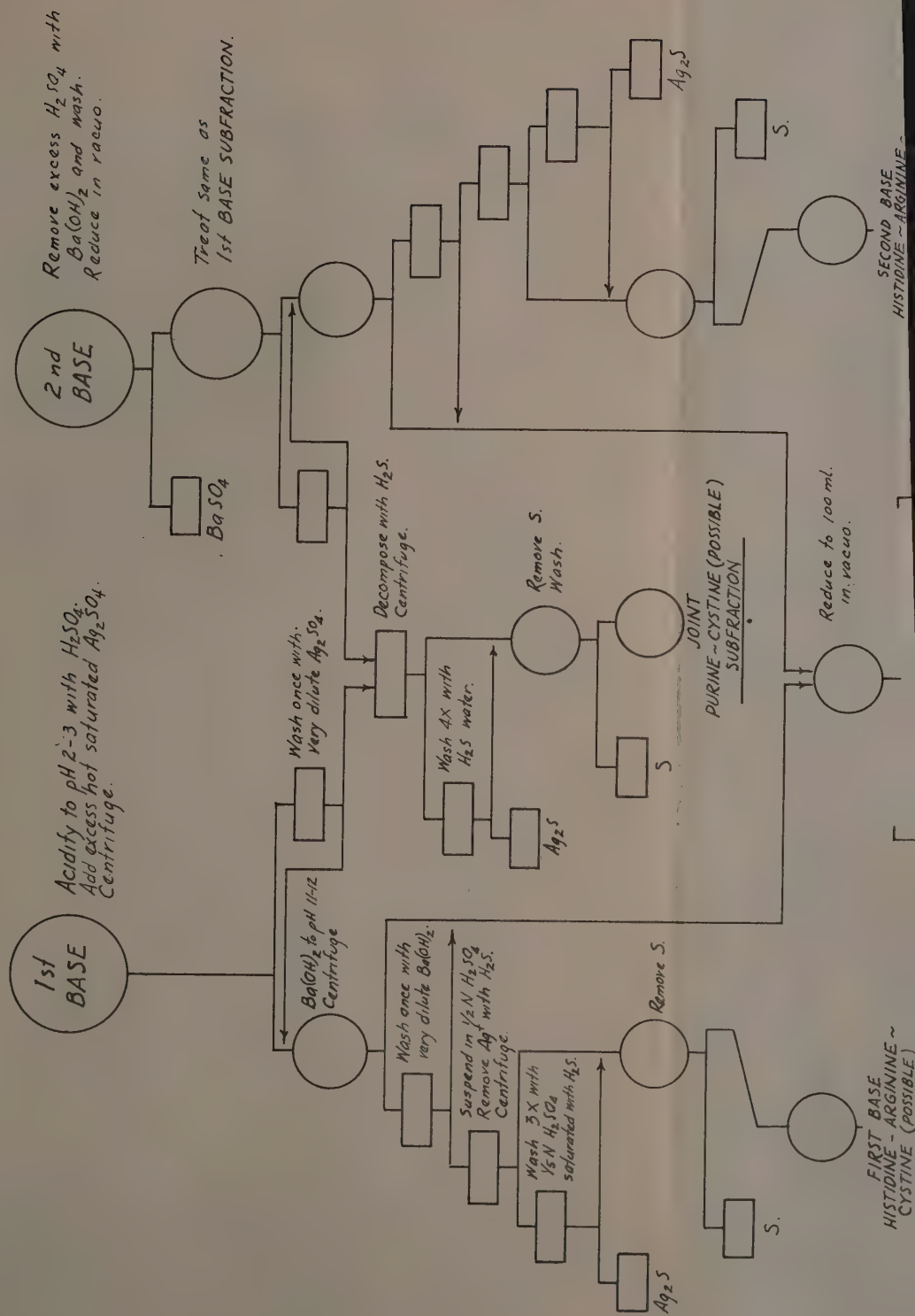
Lysine is known to be adsorbed into barium phosphotungstate precipitates, although the recovery is enhanced by dissolving this precipitate in alkali and reprecipitating in dilute solution. $BaSO_4$ precipitates also give absorption losses, particularly of cystine (29). Guanine is of such low solubility that it might be lost on any precipitate made in a small solution volume under neutral conditions.

Cystine (65, 66) arginine (49), and probably other diamino nitrogen compounds, are partly destroyed in ordinary acid hydrolysis methods. The purines, particularly guanine, are partly lost by alkali or strong acid hydrolysis (19), and only dilute

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* These might be formed from the amino pyrimidines in working a fraction down.
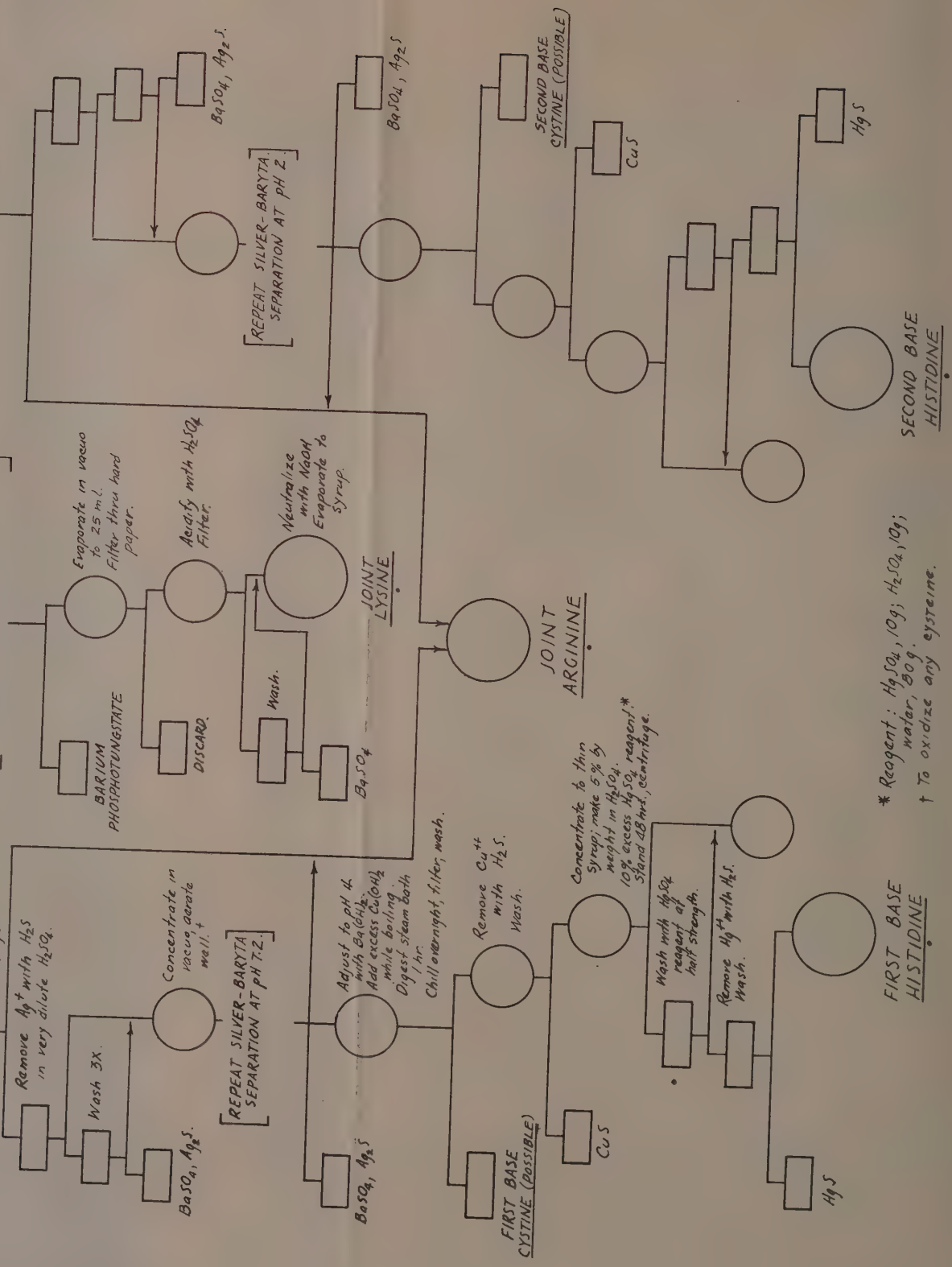
PLATE VII

RESOLUTION OF FIRST AND SECOND BASE SUBFRACTIONS

BaSO₄, Ag₂S.

[REPEAT SILVER–BARYTA SEPARATION AT pH 2.]

BaSO₄, Ag₂S

SECOND BASE (POSSIBLE)

CuS

SECOND BASE CYSTINE (POSSIBLE)

SECOND BASE HISTIDINE

HgS

Add Ag₂O until excess Ag⁺, keeping acid with H₂SO₄. Ba(OH)₂ to pH 7.2. Centrifuge.

BY THE SAME PROCEDURE USED IN PREPARATION OF FIRST AND SECOND BASE SUBFRACTIONS.

Evaporate in vacuo to 25 ml. Filter thru hard paper.

BARIUM PHOSPHOTUNGSTATE

Acidify with H₂SO₄. Filter.

DISCARD.

Neutralize with NaOH. Evaporate to syrup.

Wash.

BaSO₄

JOINT LYSINE

JOINT ARGININE

Remove Ag⁺ with H₂S in very dilute H₂SO₄.

Wash 3X.

BaSO₄, Ag₂S.

Concentrate in vacuo aerate well.†

[REPEAT SILVER–BARYTA SEPARATION AT pH 7.2.]

Adjust to pH 4 with Ba(OH)₂. Add excess Cu(OH)₂ while boiling. Digest steam bath 1 hr. Chill overnight, filter, wash.

BaSO₄, Ag₂S

Remove Cu⁺⁺ with H₂S. Wash.

CuS

FIRST BASE CYSTINE (POSSIBLE)

Concentrate to thin syrup; make 5% by weight in H₂SO₄. 10% excess HgSO₄ reagent.* Stand 48 hrs., centrifuge.

Wash with HgSO₄ reagent at half strength.

Remove Hg⁺⁺ with H₂S. Wash.

FIRST BASE HISTIDINE

AgS

* Reagent: HgSO₄, 10g; H₂O, 90g; H₂SO₄, 10g;
† To oxidize any cysteine.

acid hydrolysis permits complete recovery (25) Alkaline conditions with heat also destroy some cystine (28, 51), and racemization is even more progressive.

Cystine, histidine and particularly, arginine are incompletely precipitated in the usual phosphotungstic precipitations without special precautions, so that the commonly used correction factors are generally inadequate (50, 56, 65, 66).

The General Scheme: According to Vickery a fraction (methylated bases previously removed by Neuberg fractionation) is divided into three groups:

(1) Silver compounds insoluble in acid solution (Purine fraction)

(2) Silver compounds insoluble in alkaline solution (Histidine-Arginine fraction)

(3) Filtrate from (2) (Lysine fraction)

He adds that much further work is necessary before this fractionation can be made with certainty.

It should be pointed out that some of the difficulty encountered by Vickery *et al.* arose from unhydrolyzed complexes of the simple bases in the phosphotungstate fractions. This situation, at least, may be avoided. The possible presence of cystine was not considered in Vickery's scheme, but had it been encountered the problem would have become more complicated. The precipitation of cystine-silver begins at pH 1, is 95 per cent complete at pH 5 (65, 66) and is essentially quantitative at 5.5-6.0 (29, 65, 66). Therefore cystine would be distributed between fractions (1) and (2) in a ratio dependent upon pH during separation of fraction (1)*.

Cystine is easily separated from the other normal constituents of proteins by copper hydroxide or oxide (29). Separation of cystine from the purines (1) would be more difficult, but promise is found in the observation of Rossouw and Wilken-Jorden (40) that the cuprous mercaptide is soluble in potassium chloride.

As for the other bases, hydroxylysine, the pyrimidines and guanidine (if it survived) would probably tend to accumulate in fraction (3), because of their high solubility. Canavanine would probably accompany arginine in fraction (2), since it is precipitated by silver in alkaline solution (23).

The principles outlined were applied to the resolution of the First and Second Base Sub-fractions. The two were handled separately through certain stages, in order to note any outstanding differences that might appear. The detailed procedure is given in Plate VII.

Five nominal types of sub-fractions were obtained, the questionable position of any cystine present being indicated:

(1) Purine-Cystine (possible)

(2) Lysine

(3) Arginine

(4) Cystine (possible)

(5) Histidine

No obvious difference between the First and Second Base Sub-fractions was noted, except that the Second contained about three times as much basic nitrogen as the First. The crude sub-fractions were examined in turn for the bases theoretically represented, as follows.

---

* In a projected extension of the scheme, fraction (1) is separated at pH 2-3 to include as much as possible of any cystine, without throwing down histidine.

g. First and Second Base Histidine Sub-fractions: These fractions (Plate VII) were made to known volumes. The total nitrogen was determined in single aliquot portions, by which the whole sub-fractions were found to contain only 5 and 6 mgs. of nitrogen. Portions tested with the mercuric sulfate reagent still gave some precipitation, but no precipitate was secured with flavianic acid under conditions where histidine forms a very insoluble salt. Therefore, the traces of basic nitrogen in the fractions were due to other bases, and histidine is not present.

h. Cystine or Cysteine: The presence of this constituent in Nitrogen Fraction II was suggested earlier* (see Plate V), but no cystine or cysteine was subsequently found. Assuming a 90 per cent loss of cystine during the fractionation, 0.1 g. of nitrogen in this form in the original molasses would still have been detected. Therefore, cystine is almost certainly not present in the molasses studied.

An aliquot sample of the Purine—(possible) Cystine Sub-fraction and the First and Second Base (possible) Cystine Sub-fraction were examined. The latter two precipitates were acidified to pH 2 with sulfuric acid, treated with hydrogen sulfide, and the copper sulfide, barium sulfate and sulfur were removed and washed with very dilute hydrochloric acid. The filtrate and washings were adjusted to 25.0 ml. volumes. Samples were then subjected to the specific Sullivan colorimetric method for cystine or cysteine. All samples were negative for cystine.

i. Joint Arginine Sub-fraction: Working down this crude fraction resulted in a final fraction containing nitrogen equivalent to 93 mgs. of arginine, but from which no arginine flavianate could be isolated, showing that arginine is undoubtedly not present in the original molasses. The procedure follows:

The joint arginine fraction (Plate VII) was subjected to a second silver-baryta precipitation in very small volume, as shown in Plate VIII. The filtrate and washings (arginine residue) were saved for examination for lysine or other bases that might have been included in the initial arginine fraction.

The arginine-silver precipitate was decomposed with hydrogen sulfide in sulfuric acid solution and the barium sulfate and silver sulfide were centrifuged off and washed three times. The centrifugate and washings were freed of hydrogen sulfide and sulfur and made to 50.0 ml. volume. A total nitrogen determination was made on 1/10 of the solution and the remainder was used for precipitation of any arginine as the flavianate (56, 57).
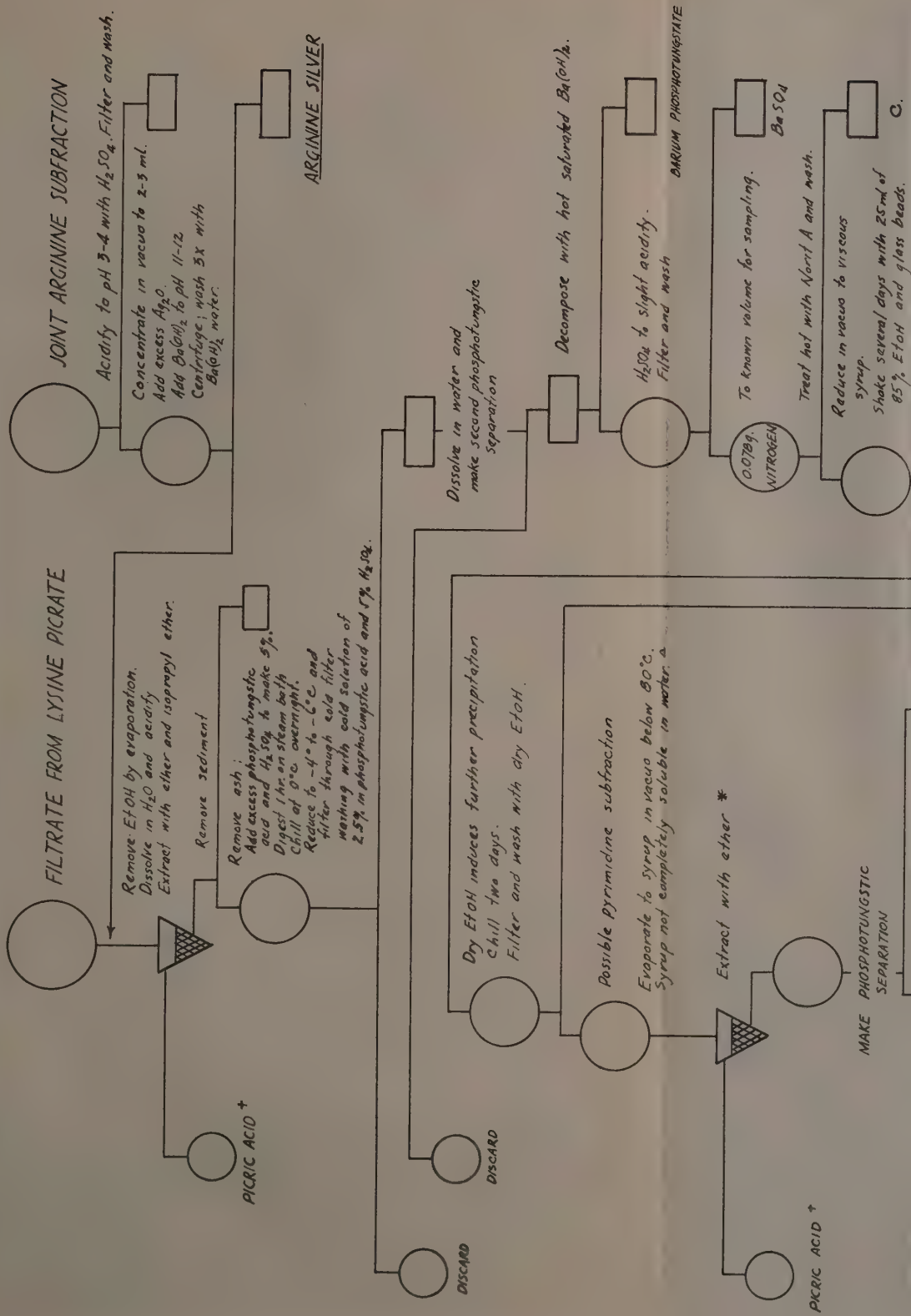
The nitrogen determination showed the remaining 9/10 of the solution to contain 21.3 mgs. of nitrogen. Precipitation of the flavianate should be essentially complete in two hours, but only a faint film of orange-colored matter (less than 1 mg.) was obtained on standing overnight. Since the probable losses of arginine during isolation cannot account for a very large proportion, arginine probably does not occur in molasses. The residual nitrogen in the final arginine fraction was too small for extensive examination for other possible bases, such as canavanine.

j. Joint Lysine Sub-fraction: Lysine picrate of high purity, equivalent to 0.30 g. of lysine was isolated. The presence of other bases, not readily forming crystalline picrates, in the residue was evident.

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* Cystine, but not methionine, liberates sulfide in hot alkaline solutions. HgS or sulfur would produce some sulfide under these conditions, but these should have been eliminated.

PLATE VIII

EXAMINATION OF LYSINE AND ARGININE RESIDUES

FILTRATE FROM LYSINE PICRATE

JOINT ARGININE SUBFRACTION

Acidify to pH 3-4 with H_2SO_4. Filter and wash.

Concentrate in vacuo to 2-5 ml.
Add excess Ag_2O.
Add $Ba(OH)_2$ to pH 11-12
Centrifuge; wash 5x with $Ba(OH)_2$ water.

ARGININE SILVER

Remove EtOH by evaporation.
Dissolve in H_2O and acidify
Extract with ether and isopropyl ether.

Remove sediment

Remove ash:
Add excess phosphotungstic
acid and H_2SO_4 to make 5%.
Digest 1 hr. on steam bath.
Chill at 0°c overnight.
Reduce to -4°c --6°c and
filter through cold filter.
washing with cold solution of
2.5% in phosphotungstic acid and 5% H_2SO_4.

Dissolve in water and
make second phosphotungstic
separation

Decompose with hot saturated $Ba(OH)_2$.

H_2SO_4 to slight acidity.
Filter and wash

BARIUM PHOSPHOTUNGSTATE

To known volume for sampling.

0.0789.
NITROGEN

$BaSO_4$

Treat hot with Norit A and wash.

Reduce in vacuo to viscous
syrup.
Shake several days with 25ml of
85% EtOH and glass beads.

C.

PICRIC ACID +

DISCARD

DISCARD

Dry EtOH induces further precipitation
Chill two days.
Filter and wash with dry EtOH.

Possible pyrimidine subfraction

Evaporate to syrup in vacuo below 80°c.
Syrup not completely soluble in water.

Extract with ether *

DISCARD

PICRIC ACID +

MAKE PHOSPHOTUNGSTIC
SEPARATION

(as lysine)
Shake intermittently for 40 hrs. Filter and wash with dry EtOH.

Remove and wash beads with hot water.

Reduce in vacuo. Dark orange to brown solid with some light yellow needles. Reddish liquid phase. Evaporate to dryness and dry over P_2O_5.

0.551 (corrected)

UNKNOWN PICRATE (DISCARDED)
[Not homogeneous M.P. determination of crude and recrystallized material shows not lysine. Accounts for about 1/3 of Nitrogen in fraction (calculated as lysine). May be picrates of pyrimidines ×]

10 ml. Add 10 ml of 5N H_2SO_4. Filter and wash.

$BaSO_4$ BARIUM PHOSPHOTUNGSTATE

Remove trace of picric acid. Add 5 ml of 6N H_2SO_4 and extract until no yellow tint in ether*

PICRIC ACID †

$BaSO_4$

Remove H_2SO_4 with $Ba(OH)_2$ until slightly acid.

Evaporate to 25 ml. for tests.

Treat with Br_2 and $Ba(OH)_2$.

10 ml

10 ml

Eliminate $BaSO_4$ masking by adding slight excess $Ba(OH)_2$ and removing with CO_2.

Treat with Br_2 and $Ba(OH)_2$ $BaSO_4$ & $BaCO_3$

Brownish-red residue.

Lavender tint not completely masked.

URACIL OR CYTOSINE

Make strongly alkaline and distill. To distillate add several drops of fresh o-amino benzaldehyde solution; evaporate to less than 3 ml, cool, acidify with HCl and make alkaline with Na_2CO_3.

BRILLIANT DISTINCT BLUE UNDER ULTRA-VIOLET RAYS
∴ 5-METHYL CYTOSINE

5 ml

Treat with Br_2. Remove excess Br_2 by boiling. Add excess $Ba(OH)_2$. (Color masked by $BaSO_4$ •)

Make strongly alkaline and distill. To distillate add several drops of fresh o-amino benzaldehyde solution. Evaporate to 1 ml. Cool, acidify with HCl and make alkaline with Na_2CO_3.

$BaSO_4$

WEAK BLUE FLUORESCENCE UNDER SCREENED ULTRA-VIOLET RAYS.

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* Picric acid interferes with cytosine-uracil test, but not with thymine -5-methylcytosine test. These tests detect 1 mg.

† Uracil ether soluble also.

× Thymine and 5-methyl cytosine form picrates. (Beilstein).

▲ Possibly anhydrides formed by alcohol and heat.

○ Color should be distinct purple salt of dialluric acid. Picric acid gives a white residue.

Following a procedure, essentially that of Vickery (56), approximately 100 ml. of ethanol were added to the syrup (Plate VII), and apparent solution was effected by prolonged mechanical shaking. Assuming a maximum of 1.5 g. of lysine, 100 per cent excess of picric acid solution (4.5 g./100 ml., in 95 per cent alcohol) was added and the shaking was continued. After standing overnight only a small fine-grained, yellow precipitate was obtained. Two days standing at room temperature gave little or no increase, but two more days at 0° C. brought down considerably more precipitate of the same appearance. This did not redissolve appreciably on shaking after raising to room temperature. Microscopically, the mass appeared as fine yellow needles or rods with a larger proportion of nodules, some of them aggregates of very fine crystals.

The picrate was separated by sintered glass filter, washed five times with dry ethanol, taken up in hot water, slowly evaporated down and dried over phosphorus pentoxide, giving a weight of 0.77 g. The product consisted of short, silky, yellow needles. Some slight contamination with inorganic salts was evinced by a few reddish streaks through the mass. A melting point determination from one spot in the mass gave a fairly sharp melting point at 267° C. (not corrected). The whole crop was dissolved in water, filtered and recrystallized, after which the melting point was lowered slightly to 263-4°, evidently due to distribution of the impurity throughout the sample. Known picrate, prepared from Eastman White Label d-lysine dihydrochloride, melted sharply at 266° C., checking the value in the literature. Neither the unknown nor the known picrate detonated until heated well above the melting point. The melting point of the 0.77 g. of isolated picrate establishes its purity. Freedom from contamination with arginine is evident. Vickery's experience shows that fairly pure picrate melts above 250° and generally above 260°.

The successful isolation of the lysine picrate in the presence of such a large excess of picric acid is probably due to the presence of other bases, which tend to react with picric acid. There was a tendency toward further slight precipitation from the alcoholic mother liquor. Therefore, the residue was worked down, along with the Arginine Residue (see next section), but evidently all of the lysine had been isolated by the first precipitation.

k. The Lysine and Arginine Residues: These residues, after joining and freeing of picric acid, non-basic nitrogen and inorganic salts, contained only 78 mg. of nitrogen. Unidentified picrates were obtained (0.35 g.), in which neither arginine nor lysine were evident. The product was expended before examination for pyrimidine bases, but the final residue from the mixed picrates was examined for the pyrimidine bases. The presence of 5-methylcytosine was confirmed, and cytosine or uracil was strongly indicated.

The detailed procedure is given in Plate VIII. The arginine residue was first worked alone to isolate lysine, but high ash in the sample interfered. Therefore the residues were united and subjected to a double precipitation with phosphotungstic acid*. The nitrogen in the fraction was determined on an aliquot portion. A picric acid separation was then made, using picric acid just equivalent to the nitrogen, calculated as lysine. The solid obtained was dissolved in hot water, reduced in vacuo and dried, but yielded only a non-homogenous glassy mass, 0.35 g. in weight. The

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* H_2SO_4 used in place of HCl, to avoid contamination of the sample with chloride.

product melted at once on a starting at 230° C., and did not detonate at any temperature. In an attempt to fractionate it, the crop was put into water solution again and reduced in volume very slowly. A brown-colored material was removed first and worked down separately to a non-homogenous yellow glass, which started melting at about 155° with softening and great swelling.

As a second crop, another non-homogenous mass was obtained. The lighter colored areas in the sample started melting around 195° with swelling and decomposition. None of these products detonated at any temperature.

The alcoholic mother liquor from the 0.35 g. of mixed picrates was carefully purified before further examination.

The pyrimidines are isolated with difficulty even from nucleic acids, but there are the tests of Johnson and coworkers for cytosine and/or uracil (69) and thymine and/or 5-methylcytosine (16). The application of these showed 5-methylcytosine to be definitely present, since thymine is reportedly absent in plants. The cystosine-uracil test indicated the probable presence of one of these, but the reaction was weak and could not be considered conclusive. Of the two, cytosine is the more probable constituent, since it would be expected in Nitrogen Fraction II.

l. The Purine-Cystine (Possible) Sub-fraction: The resolution of the Purine subfraction (Plate VII) was relieved of some complications when cystine was found to be absent. The scheme was adapted from the principles of the improved Hitchings-Fiske (18) purine group separation and the nucleic acid studies of Jones (21), Levene (27) and others. While the nucleic acid analyses employed large samples, the principles were applied here on a micro scale with fair success. With a somewhat larger sample several final products, only a fraction of a milligram in weight, could have been studied further.

Only 0.151 g. of mixed purine hydrochlorides was isolated. This, on resolution, was found to consist mostly of guanine, a very small proportion of xanthine, and an equally small amount of what was probably hypoxanthine. Adenine and uric acid were apparently absent.

The detailed procedure is given in Plate IX A and B. The product termed "Probable Hypoxanthine" was definitely not adenine, guanine or xanthine. It gave a negative murexide test and otherwise behaved like hypoxanthine, except that, in the amount isolated, it could not be obtained in recognizable crystalline form.

Conclusions:

The data on the nature of molasses nitrogen are summarized in Table XII. The actual figures are not so significant as the magnitudes they indicate. About 34 per cent of the nitrogen is in highly complex form, which leaves 66 per cent in relatively simple compounds. A similar figure is secured, if we consider the behavior of molasses solution on treatment with Amberlite IR-1 cation exchange resin. An average of 29 per cent is apparently not basic enough to be taken up by the resin, and is in simple enough form that it is not bound by adsorption. If this is added to the average of 35 per cent in the form of simple compounds recoverable on regeneration, we obtain 64 per cent for the simple nitrogen substances.

Of the two thirds of the nitrogen in relatively simple compounds, about half can be accounted for in fairly well characterized groups.

PLATE IX A

SUBFRACTIONATION OF NITROGEN FRACTION II CONTINUED RESOLUTION OF PURINE -(POSSIBLE) CYSTINE SUBFRACTION

JOINT PURINE-CYSTINE SUBFRACTION

Solution strongly acid. Filter off sludge and wash.
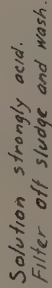
Make to 500.0 ml. Take 5.0 and 10.0 ml aliquots for tests.

NEGATIVE MUREXIDE TEST (SULFUR)

Reduce to 5 ml in vacuo; neutralize with NaOH; make slightly acid with H_2SO_4; put on steam bath and while stirring add alternate 0.15ml portions of saturated $NaHSO_3$ and 0.10ml portions 10% $CuSO_4$ until dirty white precipitate takes on yellow color. Centrifuge and wash 2X with hot water.*

NEGATIVE MUREXIDE TEST (SULFUR)

CYSTEINE - CYSTINE NEGATIVE (Sullivans method)

MUREXIDE TEST, POSITIVE URIC ACID TEST, NEGATIVE (Indicates guanine or Xanthine)

MOST OF COLOR OF FRACTION

Suspend in 12ml of 3N HCl. Decompose hot with H_2S. Filter; wash with 0.1N HCl.

CuS

Evaporate to dryness in vacuo +

Slightly yellow residue, about 0.1g. Take up in hot water. Reduce to faint acidity with NaOH.

Make second copper-bisulfite separation, reducing $NaHSO_3$ portions to 0.10ml and wash 2X with hot water

COLOR

Decompose and wash CuS as before.

Evaporate in vacuo under 60°C. to 10ml and continue at 40°C. to 4-5 ml. Add HCl to dissolve any solid. Filter off sulfur haze with 0.1 N HCl.

CuS

Concentrate to remove NH_4OH. (Further granular precipitate) Filter and cold water-wash.

Warm with 1% NH_4OH and chill (mostly soluble) Filter and wash.

Dissolve in minimum volume

Drive off NH_3. Chill overnight adjust to pH 7

XANTHINE FRACTION

SULFUR

Evaporate and dry at 40°c under atmospheric pressure to constant weight.

MIXED PURINE HYDROCHLORIDE

Dissolve in minimum volume hot dilute HCl. Neutralize with concentrated NH4OH until definitely alkaline. Section filter. white granular precipitate.

0.151 g. (CORRECTED)

Wash with 1% NH4OH. (Some dissolves)

Dissolve in minimum volume hot 5% HCl

Chill overnight. Filter and wash.

Neutralize with NH4OH. Chill overnight, filter and wash.

GUANINE
(Needles like known guanine hydrochloride. Positive murexide test.)

Dissolve in minimum volume HCl. Work down for guanine hydrochloride. Small crop.

DISCARD

DISCARD

Treat with warm ether. △

TRACE OF SOLID

Suspend in 5-7ml boiling H2O. Add saturated picric acid slowly (no immediate precipitate, unlike adenine) Chill 2 days to get clusters of short needles. Filter and wash.

Strongly acidify and extract with ether.

Suspend in small volume of boiling water (much not soluble) Precipitate reforms uncolored granular matter.○

∴ NOT ADENINE

Dissolve in HCl and extract with ether.

Neutralize with NaOH. No slightly soluble matter on evaporation.

PICRIC ACID

PICRIC ACID

Evaporate off ether, neutralize with NH4OH and chill (no immediate separation of granular guanine) Evaporate to ⅓ volume, make alkaline with NH4OH. Add ammoniacal AgNO3 ▽ Filter and wash (Bulk of precipitate is AgCl)

∴ ANY PURINE SEPARATED AS PICRATES

PICRIC ACID

DISCARD

Boil 1 min with HNO3 of 1.15 sp. gr. Filter hot and wash with HNO3

AgCl

HYPOXANTHINE FRACTION

---

* Hitchings - Fiske method.

† Temperature below 40°C on nearing dryness. Concentrated HCl above 40°C tends to deaminate adenine and guanine, or hydrolyze hydrochlorides.

× Dense precipitation at pH 5. Some dissolve in alkaline solution.

△ Hypoxanthine reported soluble and adenine insoluble.

○ Picrate behaves unlike adenine, but like guanine; no immediate picrate precipitate, short needle clusters only on standing, and needles hydrolyzed in boiling water to granular free base.

▽ 2.6 g. AgNO3 dissolved in water, plus NH4OH to redissolve initial precipitate and make to 100ml.

PLATE IX B

SUBFRACTIONATION OF NITROGEN FRACTION II, CONTINUED RESOLUTION OF PURINE-(POSSIBLE) CYSTINE SUBFRACTION

DISCARD

(Few crystals like guanine-HCl. Faint Murexide Test)

Filter and cold wash.

∴ GUANINE OR XANTHINE

BaSO₄

Evaporate in vacuo to white residue.

Make alkaline with NH₄OH. Ammoniacal AgNO₃ to maximum precipitate. Filter and wash. S

Boil 1 min. with HNO₃ sp.gr. 1.1 Filter, wash with hot HNO₃. Cool, let stand overnight. Filter, wash.

Not completely soluble in hot H₂O. Treat with H₂S. No further Ag₂S). Filter.

Treat with hot dilute H₂SO₄. Filter (some not soluble)

DISCARD

Neutralize with Ba(OH)₂. Filter.

Evaporate to small volume, make alkaline with NH₄OH (white granular precipitate) BaSO₄. Heat to remove excess NH₄OH. Separate small amount of solid.

Warm with 5% HCl and let stand. Few needles

PROBABLE GUANINE

(Opalescent filtrate). Add dilute H₂SO₄, boil and let stand (diphloematic). Filter.

DISCARD

1-2 mg white solid. No characteristic xanthine crystals. Positive Murexide test.

S

PROBABLE XANTHINE   Solubility shows not guanine.

Decompose hot with H₂S.

Make ammoniacal with NH₄OH. and filter gelatinous precipitate.

Make alkaline with NH₄OH. Filter. Ag S

Concentrate in vacuo. (Brown amorphous particles) S

Concentrate again

Some microscopic needles. Positive Murexide test. No characteristic nitrate crystals when treated with HNO₃. Many crystals like xanthine.

∴ NOT HYPOXANTHINE.

DISCARD

Suspend in H₂O and decompose with H₂S. Heat and filter.

Treat gelatinous precipitate with H₂S. Filter hot.

Evaporate in vacuo. Trace of yellow precipitate. Ag S

Cool and let stand. Yellow amorphous precipitate. Strong positive murexide test. Evaporate to small volume. Add NH₄OH and evaporate slowly; Countless small characteristic crystals of xanthine. (Very small amount)

XANTHINE

DISCARD

** Hypoxanthine. AgNO₃ insoluble in cold (Hawk & Bergeim)
†† Both give positive Murexide test. Guanine nitrate forms elongated fiberlike needles.
xx Reagent described in plate VII, footnote.

TABLE XII
SUMMARY
MOLASSES NITROGEN

1. THE DISTRIBUTION OF NITROGEN IN MOLASSES

Constituent or group	Part of molasses as nitrogen (%)	Part of total nitrogen (%)
Insoluble Nitrogen	0.0095	1.5
Protein	0.015	0.9
Polypeptide Complexes	....	31.6
Complex Nitrogen, total		34.0
Simple Nitrogen Compounds 66 (by difference)		
Nitrate Nitrogen	0.01	1.6
Ammonia	0.005-6	0.95
Alpha-amino Nitrogen	0.058	9.2
Non-amino or Proline group	....	9-11
Methylated Base group	....	0.33
Basic Amino acid-Purine-Pyrimidine group	....	3.2
Relatively Simple Amide Nitrogen*	....	5.8
Simple Compounds accounted for		30-32

2. NITROGEN COMPOUNDS ISOLATED AND IDENTIFIED

Compound	Relative amount
Proline-like Substance	..
Aspartic Acid	10
Glutamic Acid	10
Lysine	2
Unidentified bases	>1
Purines	1
Guanine (About 90%)	
Xanthine (" 5%)	
Hypoxanthine ‡ (" 5%)	
5-methylcytosine	..
Cytosine or Uracil ‡	..
Histidine	absent
Arginine	absent
Cystine	absent
Adenine	probably absent
Glycine	absent **

* Total Amide is 14.5%, including that in complexes.
  The nitrogen in the amide group only is measured.
‡ Identification not certain.
** May have been destroyed in preparation of Nitrogen Fraction II.

From the magnitudes of the simple Amido Nitrogen (5.8 per cent total nitrogen) and the alpha-amino nitrogen (9.2 per cent), we should not expect to find more than 3-4 per cent of the nitrogen in the form of free amino acids other than dicarboxylic acids.

The dicarboxylic acids or their amides (free and combined) form the major constituent, since the 14.5 per cent total nitrogen as amide nitrogen represents 29 per cent total nitrogen in amido substances.

The individual nitrogen compounds isolated from or identified in molasses, and those noted to be absent are listed in Table XII. A rough ratio between certain constituents has been set up to indicate the relative magnitudes.

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Sugar Prices

96° CENTRIFUGALS FOR THE PERIOD
DECEMBER 16, 1945, TO MARCH 15, 1946

Date	Per pound	Per ton
Dec. 16, 1945—Feb. 9, 1946	3.75¢	$75.00
Feb. 10, 1946—Mar. 15, 1946	4.205¢	$84.10

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